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The Journal of Biochemistry, Vol. 46, No. 1, 1959

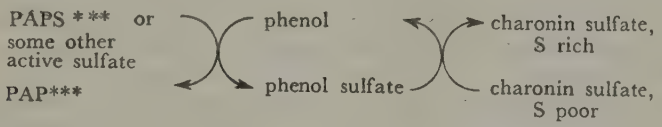
PARA-NITROPHENYL SULFATE HYDROLYSIS AND TRANSSULFATION TO CHARONINSULFURIC ACID BY CELL-FREE EXTRACTS OF "CHARONIA LAMPAS"

BY SAKARU SUZUKI, NORIKO TAKAHASHI AND FUJIO EGAMI

(From the Department of General Education and the Department of Chemistry, Nagoya University, Nagoya)

(Received for publication, March 4, 1958)

Our previous studies (*1*, *2*) have shown that radioactive sulfur was localized in the mucous gland of *Charonia lampas* (*Bosyubora*)* as the ethereal sulfate-S of charoninsulfuric acid** after the injection of S^{35}-sulfate. While it was also demonstrated that S^{35}-sulfate was incorporated into charoninsulfuric acid by slices of the mucous gland, no such incorporation was observed in extracts of acetone dried powder. However, the sulfation of charoninsulfuric acid was catalyzed by the acetone powder, when *p*-nitrophenyl S^{35}-sulfate was present as a sulfate donor in an appropriate incubation mixture (*4*). These results together with those from Lipmann's studies (*5*, *6*) on the sulfate transfer from "active sulfate" to phenol led us to consider the following mechanism and the participation of sulfatases as being consistent with the sulfation of charoninsulfuric acid (*4*).



However there are many other possibilities, as will be described later, on the sulfate transfer mechanism. So in order to investigate the possibility of the proposed hypothesis, the purification of sulfatases and the sulfate transfer enzyme system is now carried out.

* A marine gastropod, called "*Bosyubora*" in Japanese, was identificd with *Charonia lampas* (Linné), however according to T. Kira it should be identified with *Tritonalia sauliae* (Reeve).

** Charoninsulfuric acid is a mixture of glucan polysulfates with different sulfur contents (1–20 per cent). A part of the glucan has a cellulose structure and the other part an amylose structure (*3*). Cells of the mucous gland contain large amounts of these charoninsulfuric acids.

*** PAPS represents adenosine-3′-phospho-5′-phosphosulfate and PAP 3′-phosphoadenosine-5′-phosphate.

The present report deals with *p*-nitrophenyl sulfate hydrolysis and transsulfation to charoninsulfuric acid by cell-free extracts of *Charonia lampas*.

EXPERIMENTAL

Preparation of p-Nitrophenyl S^{35}-Sulfate from Inorganic S^{35}-Sulfate—To 20 mg. conc. sulfuric acid (s. g. 1.84) labelled with 10 mc. S^{35}, which was prepared from an aqueous solution of carrier-free $Na_2S^{35}O_4$ (100 mc./ml.) by evaporating the solution and dissolving the active residue in sulfuric acid, was added 20 mg. PCl_5. The reaction mixture was warmed for a few minutes at 100°, and 40 mg. chlorosulfonic acid was added as a carrier. S^{35}-chlorosulfonic acid thus prepared was added dropwise to 0.18 ml. pyridine/chloroform mixture (1/2.47, *v/v*) with stirring and cooling in an ice-salt bath. After the addition, 60 mg. *p*-nitrophenol were added as a solution in 0.14 ml. pyridine. The mixture was shaken in a tightly stoppered flask overnight at room temperature (26°). It was essential to use only anhydrous solvents and thoroughly dried equipment in order to obtain good yields. The mixture was then made slightly alkaline by addition of 80 per cent (*w/v*) KOH, in an ice bath, with efficient stirring. The mixture, to which 70 mg. potassium *p*-nitrophenyl sulfate was added as a carrier, was suspended in 3 ml. ethanol and allowed to stand overnight at 0°. The precipitate was centrifuged out, washed with 2 ml. cold ethanol and dissolved in 5 ml. 90 per cent aqueous ethanol (*v/v*) by boiling. While hot, insoluble material was rapidly centrifuged off and discarded. By cooling for a few minutes, the supernatant deposited yellow cristals which were centrifuged out, washed with 2 ml. cold ethanol to remove remaining *p*-nitrophenol, and recrystallized from 4 ml. acid aqueous ethanol (3.6 ml. ethanol plus 0.4 ml. 50 per cent acetic acid), The crystals were washed with 2 ml. cold ethanol, dissolved in 1.2 ml. hot water and reprecipitated by adding 0.6 ml. 80 per cent KOH while cooling. The resulting precipitate was centrifuged out, washed twice with 1.5 ml. cold ethanol and dried *in vacuo* over P_2O_5.

The compound was free from Cl^- and $S^{35}O_4^{--}$, but contained small traces of the parent phenol. This state of purity was sufficient for the present study. Further recrystallization from acid ethanol gave a product apparently free from *p*-nitrophenol. The yield was 70 mg. The specific radioactivity of the preparation (0.5 μc./μM) was suitable for the experiments describdd below when it was utilized as a substrate at a final concentration of 0.008–0.012 *M*.

Preparation of Acetone Powder—Fresh mucous gland was frozen and 100 g. portions were thrown into 500 ml. acetone at 0° in a chilled Waring blendors; the mixture was homogenized for 1 minute, filtered and washed successively with 500 ml. portions of acetone at −5°. After sucking dry at the pump the filter cake was broken up and dried *in vacuo* over P_2O_5 for about 10 hours. The yield was approximately 20 g.

Assay of Arylsulfatases—The assay mixture contained 40–80 μg. *p*-nitrophenol in 1 ml. After incubation, the reaction was stopped by the addition of 0.2 ml. 1 per cent aqueous trichloroacetic acid followed by 1.8 ml. ethanol. After centrifugation, a 2 ml. portion of the supernatant was pipetted into 2 ml. 1 *M* Na_2CO_3 solution. The intensity of the yellow colour was read at 400 mμ against a suitable blank. Assays were performed in duplicate and the appropriate enzyme and substrate blanks were always included.

Preparation of the "Crude Extract" of Mucous Gland—10 per cent (*w/v*) suspension of acetone dried mucous gland in 2 per cent KCl solution containing 0.1 per cent (*v/v*) chloroform was incubated at 36° for 24 hours and then centrifuged. The supernatant was dialyzed overnight at room temperature against running water and insoluble material was removed by centrifuging. To the supernatant was added chilled acetone, giving a final

acetone concentration of 60 per cent (*v/v*). The precipitate obtained from 10 g. acetone powder was dissolved in 20 ml. distilled water. This preparation ("crude extract") contained a considerable amount of charonin sulfates with 0–5 per cent sulfur contents, and gave a reddish colour reaction with both toluidine blue and iodine reagents.

Fractionation of Enzyme by Electrophoresis—Electrophoresis, employing a slab of potato starch as a supporting medium, was carried out in an apparatus developed from that of Larson *et al.* (*7*). The starch slab with a gutter in the middle, 1 cm. wide, was perfused with an appropriate buffer for 4 hours. Then the gutter was filled to the brim with starch paste containing the enzyme preparation which was previously concentrated by acetone precipitation or freeze-drying. Electrophoresis was carried out at room temperature for 17 hours in the presence of veronal buffer, pH 8.6, tris-acetate buffer, pH 7.0, or acetate buffer, pH 6.0. The starch was then cut into sections, 1 cm. wide, which were each suspended in 10 ml. water and allowed to stand for several hours or overnight, as was convenient, in an ice box to obtain a clear supernatant. The other details are described in reference to each experiment.

Preparation of Liver Arylsulfatase—A 17 per cent (*w/v*) suspension of acetone dried liver or frozen liver in water containing 0.1 per cent (*v/v*) chloroform was homogenized in a Waring blendor, incubated at 30° for 24 hours and then centrifuged. To the supernatant was added acriflavin (Taypaflavin) to a final concentration of 1 per cent (*w/v*). The resulting precipitate was centrifuged off and discarded. The dialyzed supernatant was brought to 50 per cent saturation with solid $(NH_4)_2SO_4$ at pH 6.0; the inactive precipitate was centrifuged off, and supernatant was saturated with $(NH_4)_2SO_4$. After 16 hours, the active precipitate was centrifuged off, dissolved in water and dialyzed overnight. Further purification was carried out by zone electrophoresis on a starch slab. The active fraction was dissolved in water and dialyzed overnight. The enzyme in the dialyzate was then adsorbed on IRC-50, eluted with 2 *M* tris-acetate buffer, pH 8, dialyzed and freeze-dried.

In a typical preparation the recovery of arylsulfatase units at the final stage was about 25 per cent of the initial extract and represented a 60 fold increase in specific activity (units/mg. protein). The preparation gave a negative Molisch's test for carbohydrate.

Paperchromatography and Autoradiography—In an earlier investigation (*1*, *4*), method involving isolation of charoninsulfuric acid in a fairly high degree of purity was used to establish the incorporation of radioactive sulfur into charoninsulfuric acid. However, the procedure was time-consuming and unsuited for serial measurements. In the present study, a rapid method, partly described in a preliminary note (*4*) and based upon the separation of charoninsulfuric acid from the other radioactive substances by means of ascending paperchromatography, was employed.

0.03 ml. test mixture was put on a filter paper (Toyo, No. 50) as a thin band and the paper was developed by the ascending technique in mixture of *n*-butanol, ethanol, water and ammonia (40, 12, 20 and 1) until the front reached 30 cm. from the original line. The counts of the paper were read at intervals of 1 cm. by means of Geiger-Müller counter to locate radioactive substances, which were visualized, if necessary, by radioautography on X ray film (exposure for 20 days at 0°).

RESULTS

Properties of "Crude Extract"—As in the case of acetone powder a liberation of *p*-nitrophenol occurred on allowing the "crude extract" to incubate with *p*-nitrophenyl sulfate.

The effect of pH on the activity was determined over the range 4.5–8.2

in 0.05 *M* tris-acetate buffer at a final substrate concentration of 0.01 *M*. When incubation was carried out for 3 hours, there was an optimum at pH 5.5 (Fig. 1).

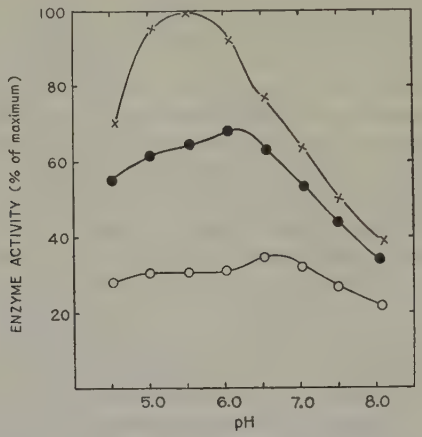


FIG. 1. Effect of pH on the liberation of *p*-nitrophenol by "crude extract". Incubated at 36° in 0.05 *M* tris-acetic acid buffers of varing pH. Substrate concentration 0.01 *M* *p*-nitrophenyl sulfate. Final volume of reaction mixture 4 ml. containing 1 ml. enzyme solution. Maximum enzyme activity; 0.1 μM *p*-nitrophenol/ml. enzyme/hour.

—○—, incubated for 1 hour; —●—, incubatedd for 2 hours; —×—, incubated for 3 hours.

As time of incubation was made shorter, the optimum shifted from pH 5.5 through 6.0 to 6.5. These results were at variance with those in the investigation using acetone powder, in which case the pH optimum for the liberation of *p*-nitrophenol was approximately 7.2 at any time of incubation during 20 hours. It is difficult to explain these anomalous relationships, but it would appear that these were brought about by the crude nature of of these enzyme preparations.

As indicated in the preliminary experiment using acetone powder, the liberation of *p*-nitrophenol was strongly inhibited by phosphate and fluoride. This together with the fact that formation of ethereal sulfates was always accompanied by the liberation of inorganic sulfate suggested that the transferase activity might be related to arylsulfatase. The action of inhibitors on "crude extract", therefore, was investigated. Like most arylsulfatases, this enzyme system was inhibited by sulfite and phosphate (Table I). It was also inhibited by sulfate and fluoride but was unifluenced by cyanide. The activation by glucose is also worthy of note since it was shown that arylsulfatase in the liver of *Charonia* was activated by glucose (*8*). A similar activation was observed in the presence of maltose or cellobiose, but no effects were exerted by polysaccharides. Charoninsulfuric acid with high sulfur contents, however,

exhibited a inhibitory action which is presumably due to coprecipitation of the enzyme with the charoninsulfuric acid.

TABLE I

Effect of Various Compounds on the Activity of "Crude Extract" Measured by the p-nitrophenol Liberation

Final volume of reaction mixture 4 ml. containing 2 ml. 0.1 *M* tris-acetic acid buffer, pH 6.5, 1 ml. 0.04 *M* *p*-nitrophenyl sulfate and 1 ml. enzyme solution. The inhibitor and activator were dissolved in the buffer to give the required concentration. Incubation for 8 hours at 36°.

Compound	Final concentration	Inhibition (−) or activation (+)
Na_2SO_4	0.01 *M*	−13 %
Na_2SO_3	0.01	−80
KH_2PO_4	0.01	−80
NaF	0.01	−52
KCN	0.01	0
Glucose	3.0 %	+24
Cellobiose	3.0	+17
Maltose	3.0	+11
Amylose	3.0	0
Glycogen	3.0	0
Carboxymethyl cellulose	3.0	0
Charonin sulfate S: 2%	3.0	− 5
S: 15%	saturated	−24

Transferase Activity of "Crude Extract"—As shown in Fig. 2 three radioactive substances coresponding to charoninsulfuric acid, inorganic sulfate and glucose monosulfate appeared at the levels with R_f values of 0.00, 0.09 and 0.19 respectively. No reaction took place when the *p*-nitrophenyl S^{35}-sulfate was substituted by $K_2S^{35}O_4$ or the enzyme preparation was denaturated by heat. These results indicate that the "crude extract" as the acetone powder has an enzyme system catalyzing a sulfate transfer from *p*-nitrophenyl sulfate to carbohydrate. The fact that such sulfate transfer was always accompanied by hydrolysis is suggestive of the possibility that arylsulfatase might participate in both reactions, since the transferase activity of hydrolytic enzymes has been shown to be quite common. (*e.g.* transferase activity of phosphatases (*10*)).

Moreover the extremely high activity of arylsulfatases and carbohydrate sulfatases in this organism, and especially the coexistence of highly active arylsulfatases and charonin sulfate in its mucous gland also incite us to the working hypothesis that these sulfatases may participate in the transfer

reaction.

Further results presented in Fig. 2 on the action of inhibitors however were not in accord with the simple reasoning that arylsulfutase alone may

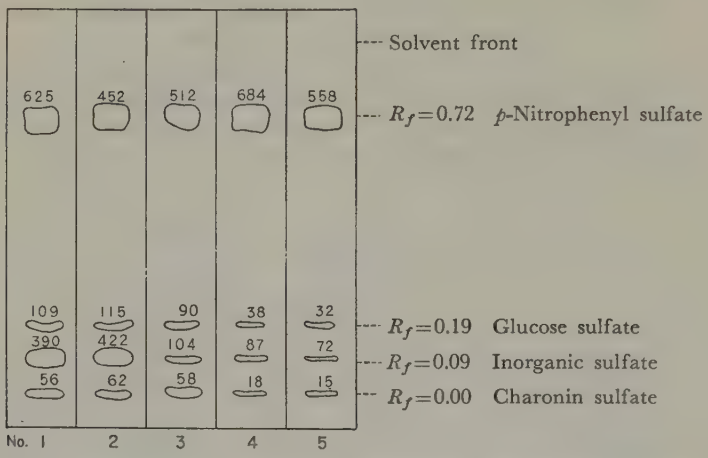


FIG. 2. Detection of S^{35}-containing products by paper autoradiography. Final volume of reaction mixture 0.4 ml., containing 0.1 ml. crude extract, 0.1 ml. 0.04 *M* *p*-nitrophenyl S^{35}-sulfate, 0.1 ml. 0.2 *M* tris-acetic acid buffer pH 7.0 and each 0.1 ml. water (No. 1), 0.5 *M* glucose (No. 2), 0.04 *M* NaF (No. 3), 0.04 *M* KH_2PO_4 (No. 4) or 0.04 *M* Na_2SO_3 (No. 5). Incubated for 20 hours at 36°. Solvent: *n*-butanol/ethanol/water/ammonia, 40/12/20/1. Values are expressed as c.p.m. of each spot on chromatograms.

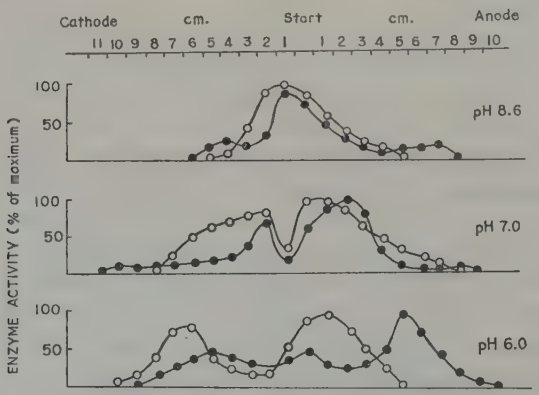


FIG. 3. Electrophoretic movement of "crude extract". 2.5 ml. portions of "crude extract" were taken for electrophoresis. Protein was located by Folin's reaction and enzyme activity was assayed by measuring liberated *p*-nitrophenol.

—●—, protein: —○—, enzyme activity.

catalyse the transfer reaction. For instance, the formation of ethereal sulfates was not affected by 0.01 *M* fluoride, while that of inorganic sulfate was

strongly depressed under the same conditions.

Fractionation of "Grude Extract"—In order to answer the question whether or not arylsulfatase participates in sulfale transfer, further fractionation of the "crude extract" was carried out.

Electrophoresis using starch slab suggested that considerable purification was possible by the use of this technique. In these investigations, protein located by Folin's reaction and the enzyme was located by incubating each fraction with 0.01 *M* *p*-nitrophenyl sulfate in 0.05 *M* acetate buffer at pH 6.0. When the electrophoresis was run at pH 8.6, there was no indication of separation, *i.e.* only one peak of the enzyme avtivity overlapping the peak of protein was obtained. However, two well-defined peaks of enzyme activity were separated during 16 hours at 300 v. in the presence of 0.05 *M* acetate, pH 6.0–7.0 (Fig. 3). One enzyme migrated towards the anode (sulfatase I) and another towards the cathode (sulfatase II) at pH 6.0.

Both enzyme preparation still contained charoninsulfuric acid with low sulfur contents.

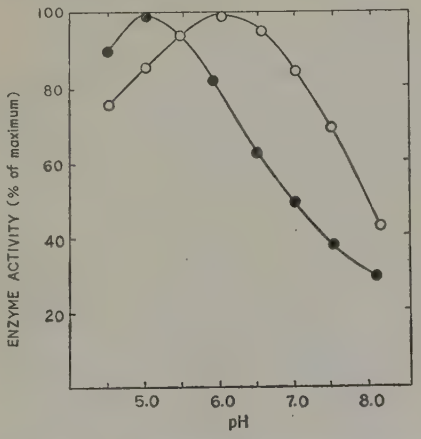


FIG. 4. pH-activity curves for sulfatases I and II. Incubated for 8 hours at 36° in the presence of 0.05 *M* tris-acetic acid buffer adjusted to the required pH; general conditions as in Fig. 1.

Maximum enzyme activity: 0.1 (I), 0.08 (II) μM *p*-nitrophenol/ml. enzyme/hour.

—○—, sulfatase I; —●—, sulfatase II.

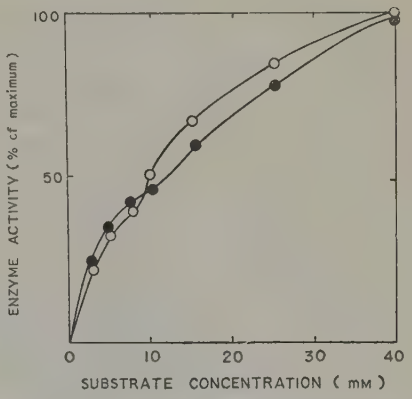


FIG. 5. Substrate concentration-activity curves for sulfatase I and II. Incubation was for 8 hours at 36° in the presence of 0.05 *M* tris-acetic acid buffer adjusted to the appropriate optimum pH (See Fig. 4).

Maximum activity: 0.2 (I), 0.16 (II) μM *p*-nitrophenol/ml. enzyme/hour.

—○—, sulfatase I; —●—, sulfatase II.

Fig. 4 indicates that the pH optima for sulfatases I and II were respectively 6.0 and 5.0 in 0.05 *M* tris-acetate buffer at a final substrate concentration of 0.01 *M* *p*-nitrophenyl sulfate.

Fig. 5 shows the effect of substrate concentration on sulfatase I and II. With both enzymes, maximum activity was not reached at 40 mM *p*-nitrophenyl sulfate. The liberation of *p*-nitrophenol was inhibited to 90 per cent by

0.01 *M* KH_2PO_4, to 85 per cent by 0.01 *M* Na_2SO_3 and to 80 per cent by NaF in the case of sulfatase I, and to 90 per cent by KH_2PO_4, Na_2SO_3 and NaF in the case of sulfatase II.

Autoradiographic investigation on these enzyme indicate that no sulfate

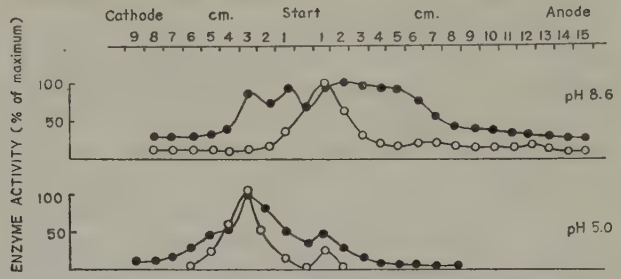


FIG. 6. Electrophoretic movement of liver extract. Protein was located by Folin's reaction and enzyme activity was assayed by measuring liberated *p*-nitrophenol.

—○—, protein; —●—, enzyme activity.

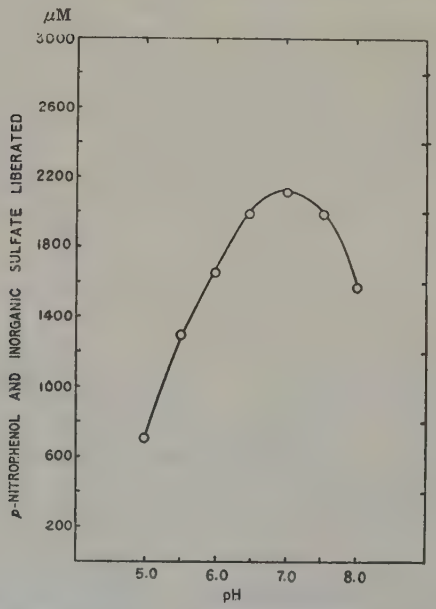


FIG. 7. pH-activity curve for liver arylsulfatase. Incubation 3 hours at 36° in the presence of 0.07 *M* tris-acetate buffer or acetate buffer adjusted to the required pH. Substrate concentration 0.003 *M* *p*-nitrophenyl sulfate. Final volume of reaction mixture 3 ml. containing 1 ml. enzyme solution.

glucose or 0.36 per cent (*w*/*v*) charoninsulfuric acid. For instance, 466 c.p.m. (sulfatase I) or 662 c.p.m. (sulfatase II) of inorganic sulfate was transfer from *p*-nitrophenyl sulfate occurred, even on addition of 0.05 *M*

liberated from 1,500 c.p.m. of *p*-nitrophenyl S^{35}-sulfate for 16 hours but no radioactive substances corresponding to charaninsulfuric acid or glucose sulfate were observed. (Thses values are expressed as counts of each spot on chromatogram). These results however do not necessarily show that different enzymes are responsible for sulfate transfer and hydrolysis, since there may be some other additional factors necessary for the formation of charoninsulfuric acid.

Transferase Activity of Liver Arylsulfatase—The further investigation on the transferase activity of arysulfatase was carried out by using the liver of *Charonia* as a source of the enzyme.

This is of particular interest since this partially purified arylsulfatase was free of charoninsulfuric acid. The typical results of electrophoretic experiments are indicated in Fig. 6.

As shown in Fig. 7, the shape of pH-activity curve was different from that of arylsulfatases in mucous gland. However, there was no significant difference in the effect of inhibitors on liver- and mucous gland-arylsulfatases so far as investigated.

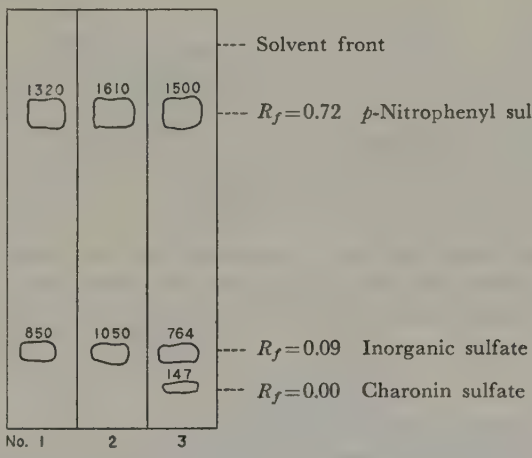


FIG. 8. S^{35} incorporation into charoninsulfuric acid by liver arylsufatase. Final volume of reaction mixture 0.2 ml., containing 0.1 ml. enzyme solution, 0.02 ml. 0.04 *M* *p*-nitrophenyl S^{35}-sulfate, 0.08 ml. 0.1 *M* tris-acetate buffer pH 7.0 (No. 1), and 1 per cent dialyzed charoninsulfuric acid (No. 2), or non-dialyzed charoninsulfuric acid (No. 3). Incubated for 3 hours at 37°.

The results on the transferase action of this enzyme are indicated in Fig. 8, which shows that charonin sulfate synthesis was catalyzed by the liver arylsulfatase preparation on addition of non-dialyzed (crude) charoninsulfuric acid with low sulfur contents. No synthesis was observed when dialyzed charoninsulfuric acid was used as acceptor. Possibly some unknown factor participates in the transfer reaction.

Without much more extensive purification both of the liver enzyme and

the factor, it is not absolutely certain that the sulfatases participate in the transfer reaction, and other possibilities can not be excluded. For example, there may be a specific transferase which transfers sulfate from *p*-nitrophenyl sulfate to charonin sulfate, or this transfer may involve the intermediation of PAP as a carrier of sulfate from *p*-nitrophenyl sulfate to charonin sulfate (*10*). Finaly, possibility must be considered that uridine nucleotides may be intermediates in the synthesis of charonin sulfate, as they were believed to be in the synthesis of chondroitin sulfate (*11*).

SUMMARY

1. A method of synthesis of *p*-nitrophenyl S^{35}-sulfate was described.

2. Cell-free extract of the mucous gland of "*Charonia lampas*" has been found to incorporate radioactive sulfate into charoninsulfuric acid when incubated with *p*-nitrophenyl S^{35}-sulfate.

3. Two arylsulfatases with different pH optimum (5.0 and 6.0) were found in the extract of mucous gland and separated by zone electrophoresis. These arylsulfatases were not able to transfer sulfate from phenol to carbohydrate.

4. Arylsulfatase of the liver of *Charonia lampas* was purified. The purified arylsulfatase preparation has been found to catalyze the incorporation of S^{35}-sulfate to polysaccharide in the presence of a non-dialyzed preparation of charoninsulfuric acid and *p*-nitrophenyl S^{35}-sulfate. This activity has not been observed with dialyzed charoninsulfuric acid. Some unknown factor may participate in the reaction.

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CRYSTALLINE CYTOCHROME C

IV. CRYSTALLIZATION OF BEEF KIDNEY CYTOCHROME C.

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In the preceding papers of this series (*1–3*) three methods were described for the preparation of crystalline cytochrome c from mammalian and fish hearts as well as from pigeon breast muscle. The occurrence of cytochrome c is, however, not confined to muscular tissues, but it is widely distributed in a variety of animal organs. Therefore, it seems of considerable interest to isolate the cytochrome from non-muscular sources and to compare it with that obtained from muscles.

The purpose of the present paper is to report a procedure to isolate crystalline cytochrome c from beef kidney, an organ which has been known as the richest non-muscular source of the pigment (*4*). Kidney tissues contain impurities which cannot be removed by any of the purification methods developed for muscular tissues. In the present work, however, this difficulty was overcome by introducing a step in which the cytochrome was chromotographed in the reduced form. A preliminary account of this work has been published elsewhere (*5*).

EXPERIMENTALS

Cytochrome c was assayed and the resin Amberlite XE-64 was treated as previously described (*1*).

For the purification and crystallization of kidney cytochrome c, two different methods were tried. Both of them gave crystalline products.

Method A

This method was essentially the same with Method I described previously (*1*), except that the solution of cytochrome c obtained by chromatography was concentrated by a resin column, reduced, and further purified by chromatography.

Step 1. Extraction—About 6 kg. of beef kidneys were freed from fat and ligaments, cut into small pieces, and washed with water. The liquid was drained through cloth and the tissues were minced in a sharp meat mincer. Mince thus obtained (4.5 kg.) were treated in the same way as Step 1 of Method I (*1*), except that 100 g. of Celite 545 were added per kg. of mince prior to squeezing. Combined the first and the second extracts,

turbid brown solution, *ca.* 8 liters, pH 6.0, 45 per cent saturated with respect to AS*.

Step 2. AS Treatment—The same as Step 2 of Method I (*1*). Combined filtrate and washings; clear, yellowish pink solution, *ca.* 8.5 liters, pH 7, 80 per cent saturated with respect to AS.

Step 3. TCA Precipitaiton—The same as Step 3 of Method I (*1*). Dialysed filtrate; clear pink solution, *ca.* 300 ml., pH 7.

Step 4. Adsorption and Elution—The same as Step 4 of Method II (*2*). Eluate; clear red solution, *ca.* 10 ml., pH 6.5.

Step 5. Chromatography of Oxidized Cytochrome—The same as Step 5 of Method II (*2*), except that a 2×20 cm. column was used. Main fraction; reddish pink solution, *ca.* 80 ml.

Step 6. Concentration by Resin Column—The main fraction from Step 5 was diluted two fold with distilled water and adsorbed onto a resin bed (2×3 cm.) placed in a glass filter. After removing the liquid by suction, the cytochrome was eluted with 0.5 *N* (0.5 g. ion NH_4^+) ammonium phosphate buffer of pH 7.0 without transferring the resin to another column (see Step 6 of Method I). Eluate; dark red solution, *ca.* 5 ml.

Step 7. Chromatography of Reduced Cytochrome—The eluate was adjusted to pH 8.5 with a few drops of 30 per cent ammonia and the cytochrome was completely reduced by adding a few mg. of ascorbic acid. After a few minutes the solution was adjusted to pH 7.5 with 1 *N* phosphoric acid and diluted 2.5-fold with distilled water (dark pink solution containing *ca.* 0.18 g. ion of NH_4^+, 13 ml.). The solution was then passed through a 2×25 cm. column of resin previously equilibrated with 0.2 *N* ammonium phosphate buffer of pH 7.5 containing 0.001 *M* ascorbic acid. The reduced cytochrome adsorbed on the top of column was further developed with the same buffer and the effluent containing the main fraction of cytochrome was collected. Main fraction; clear pink solution, *ca.* 100 ml.

Step 8. Second Concentration by Resin Column—A minimum amount of 0.01 *M* potassium ferricyanide was added to the solution obtained from Step 7 to convert all the cytochrome to the oxidized form and the solution was treated essentially as Step 6 of Method I (*1*).

Step 9. Crystallization—Essentially the same as Step 7 of Metood I (*1*).

Method B

This was a modification Method III reported in Paper III (*3*). In this experiment beef kidney was freed from medulla and only cortex was used.

Step 1. Extraction—About 7 kg. of beef kidneys, freed from fat, were cut into pieces of convenient size and the medulla part was removed as well as possible. The cortex, still contaminated by a small amount of medulla, was passed through a meat grinder. Mince thus obtained (3 kg.) were treated in the same way as described for Step 1 of Method III (*3*), except that 200 g. of Celite 545 were used per kg. of mince in squeezing. The combined 1st and 2nd extracts (*ca.* 5 liters, pH 6.0) were filtered using a Buchner funnel and 300 g. of Hyflo Super-Cel as filter aid and the residue was washed with 500 ml. of water. The filtration was considerably slow. The combined filtrate and washing; turbid colloidal solution, *ca.* 5.2 liters, pH 6.0.

Step 2. First Adsorption and Elution—The same as Step 2 of Method III (*3*), except that 10×2 cm. resin column was used. Eluate; clear, brownish pink solution, *ca.* 150 ml., containing *ca.* 0.4 g. ion of NH_4^+, pH 6.5.

Step 3. Second Adsorption and Elution—The same as Step 5 of Method III (*3*). Eluate; clear red solution, *ca.* 20 ml., containing 0.4 g. ion of NH_4^+, pH 6.5.

Subsequent five steps, *i.e.*, *Step 4. Chromatography of Oxidized Cytochrome*, *Step 5. Concentration*

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* Ammonium sulfate and trichloroacetic acid are abbreviated in this paper as AS and TCA respectively.

by Resin Column, Step 6. Chromatography of Reduced Cytochrome, Step 7. Second Concentration by Resin Column, and Step 8. Crystallization were performed in the same ways as described above for Steps 5 to 9, respectively, of Method A.

RESULTS AND DISCUSSION

Extraction of Kidney Cytochrome c—As in the case of muscles (*3*), kidney cytochrome c was equally well extracted at nearly neutral reactions in the presence (Method A) and absence (Method B) of AS if the tissue was preliminary treated with acetic acid. Squeezing of the extract suspension was quite difficult, especially in Method B, and larger amounts of filter aid were necessary than with muscles.

In order to avoid increase in volume, kidneys in Method B were freed from medulla which contains practically no cytochrome c. To remove medulla was, however, by no means an easy task.

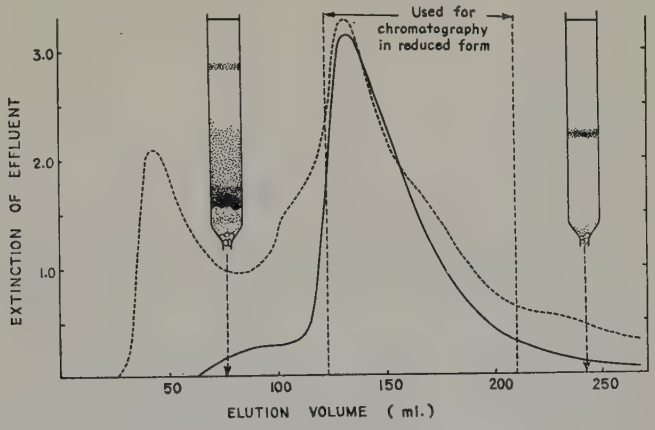

FIG. 1. Chromatography of beef kidney cytochrome c in oxidized state.

Charged cytochrome c; the one obtained at Step 3 of Method B. Column; Amberlite XE-64, 2 × 20 cm. Buffer; 0.25 *N* ammonium phosphate (0.25 g. ion of NH_4^+), pH 7.0. Dotted line; extinction at 280 mμ. Solid line; extinction at 550 mμ. Figures of columns show the chromatograms at the arrows.

In Method B the original extracts had to be filtered with great difficulty in order to effect direct adsorption of the cytochrome to the resin. Both the filtration and adsorption procedures were very troublesome and time-consuming in large-scale preparative work. In Method A, on the other hand, the pigment was quite easily collected by precipitating it with TCA even from a relatively large amount of turbid extract saving much time as well as effort.

Modification of Cytochrome—Precipitation of cytochrome c with TCA might be most responsible for the modification of its protein. In both Method A and B only a very small part of the cytochrome was converted to modified forms during the acetic acid treatment and the subsequent extraction

procedures (see Fig. 1) if the directions given were carefully followed.

Considerable modifications were, however, observed after the TCA precipitation step in Method A as can be seen from Fig. 2 which shows that the cytochrome was divided into more than 10 bands on chromatography. In this figure the main band at the bottom of column represents the unmodified pigment. It is apparent that the cytochrome underwent various degrees of modifications and that all of the modified pigments possessed higher affinities to the resin than the native one. The number of the modified cytochrome bands varied from experiment to experiment, but it was always greater as compared with that obtainable with beef heart cytochrome c treated in the same manner.

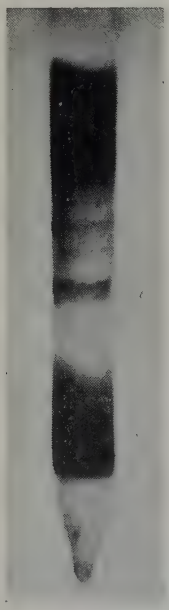

FIG. 2. Chromatogram of beef kidney cytochrome c obtained by the TCA-AS method.

Charged cytochrome c was obtained at Step 4 of Method A. The other conditions were the same as Fig. 1.

It should further be pointed out that the total amount of the cytochrome modified during the TCA treatment was much larger than can be seen from Fig. 2. The cytochrome preparation used in the experiment shown in Fig. 2 was obtained after the adsorption and elution procedure (Step 4 of Method A), and in this step the seriously modified cytochrome of strong affinities to the resin had already been removed.

Chromatography of Cytochrome in Reduced State—As already mentioned, the kidney extracts contain considerable quantities of impurity possessing a similar affinity to the resin to that of oxidized cytochrome c. The cytochrome preparation from kidney was, therefore, only about 70 per cent pure even after chromatography in the oxidized state (Step 5 of Method A or Step 4 of Method B), a procedure which resulted in sufficient purities for crystallization in the case of muscular cytochromes. With the kidney pigment,

therefore, further purification was necessary to reach a purity suitable for crystallization. This was accomplished by taking advantage of the fact that the affinity of reduced cytochrome c to the resin is lower than that of the oxidized pigment. Thus, the cytochrome was first converted to the reduced

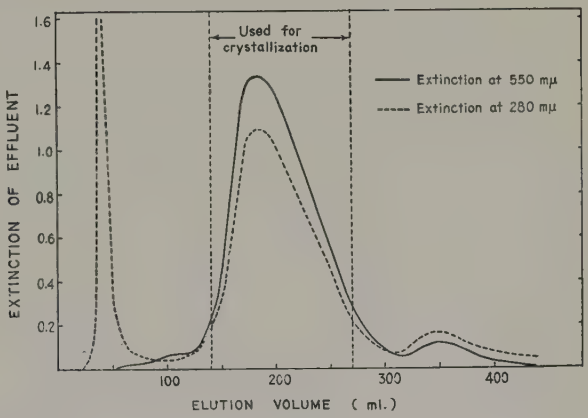

FIG. 3. Chromatography of beef kidney cytochrome c in reduced state. Charged cytochrome c; the one obtained at Step 5 of Method B was reduced with ascorbic acid. Column; Amberlite XE-64, 2 × 25 cm. Buffer; 0.20 *N* ammonium phosphate, pH 7.5 containing 0.001 *M* ascorbic acid.

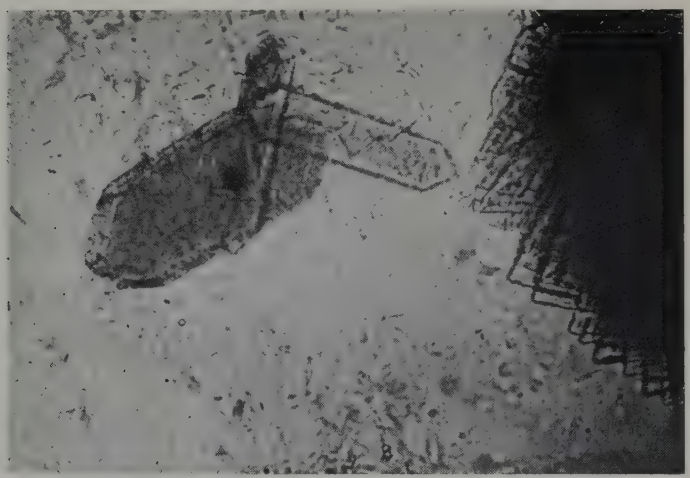

FIG. 4. Crystalline reduced cytochrome c from beef kidney. Plates and plate-rosette (× 300)

form by the addition of ascorbic acid and chromatographed on a column of the resin using the buffer at a lower concentration as described under EXPERIMENTALS.

A typical elution curve from such chromatographic experiments is shown in Fig. 3.

The cytochrome preparation used in this experiment was obtained from Method B. Essentially the same elution curve was also obtained with preparations purified by Method A. As can be seen from Fig. 3, most of the impurity protein remained under this condition still in the column when the main fraction of cytochrome c had just passed through the column. The cytochrome was thus sufficiently purified for crystallization.

TABLE I

Summary of Purification and Crystallization of Cytochrome c from Beef Kidney by Method A

Steps	Volume (*ml.*)	Yield (μ *moles*)	Purity (E_{550}/E_{280})
1. Extracts from 6 kg. kidneys	8,000		
2. AS treatment	8,500		0.02
3. TCA-AS precipitation	300	6.0	0.25
4. Adsorption and elution	10	4.8	0.60
5. Chromatography in oxidized form	80	2.8	0.90
6. Concentration of the main effluent	5		
7. Chromatography in reduced form	100	2.0	1.22
8. Concentration of the main effluent	0.5		
9. Crystallization	0.5	1.5	1.26

TABLE II

Summary of Purification and Crystallization of Cytochrome c from Beef Kidney by Method B

Steps	Volume (*ml.*)	Yield (μ *moles*)	Purity (E_{550}/E_{280})
1. Extracts from 3 kg. cortex (6 kg. kidney)	5,200		0.02
2. 1st adsorption and elution	150	6.2	0.35
3. 2nd adsorption and elution	20	5.3	0.65
4. Chromatography in oxidized form	100	4.5	0.96
5. Concentration of the main effluent	5	4.3	0.95
6. Chromatography in reduced form	120	3.8	1.20
7. Concentration of the main effluent	0.5		
8. Crystallization	0.5	3.1	1.28

The peak at 280 mμ appearing before the main peak of cytochrome c corresponds to ascorbic acid used for reduction of charged cytochrome c and not to protein impurities.

Crystallization—The reduced form of kidney cytochrome c was crystallizable from thus purified solutions in the same way as in the case of beef heart cytochrome c and the crystals usually appeared in rosette forms which resembled those of the beef heart pigment. However, if the suspension of crystals were allowed to stand at room temperature for more than six months, they sometimes changed to hexagonal plates or rosettes composed of a number of fairly large plates (Fig. 4).

Summary of Purification Methods—In Tables I and II are summarized typical results obtained with Method A and B, respectively. The yield of the crystalline cytochrome c per kg. of original kidney (about 0.5 kg. of cortex) was about 20 mg. with Method B, while it was only about 10 mg. with Method A possibly due to the modification which cytochrome c underwent during the TCA precipitation. The purity of the crystals obtained was the same in the two methods. It was also the same as that of the crystalline beef heart cytochrome c.

SUMMARY

Kidney tissues contain an impure protein which prevents sufficient purification of cytochrome c for crystallization by any of the methods reported for muscle tissues. This impurity possessing a similar affinity to the resin to that of oxidized cytochrome c was found to be removed by chromatography of the cytochrome solution in its reduced form permitting easy crystallization of the pigment. On the basis of this finding, two methods, *i.e.*, Methods A and B, were described for purification and crystallization of kidney cytochrome c. These two methods are the modifications of Method III (using direct adsorption) reported in the previous papers. In the latter method the yield of crystals was about 40 mg. from 6 kg. of kidneys (3 kg. of cortex), while the former method gave a smaller yield because of modification of the pigment caused during the TCA precipitation. Crystals usually appeared as rosettes similar to those of beef heart cytochrome c and sometimes as hexagonal plates.

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ACTION OF CHYMOTRYPSIN ON SYNTHETIC SUBSTRATES

I. ACTION OF α-CHYMOTRYPSIN ON AMINOACYL-L-TYROSINAMIDES

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Earlier studies on chymotrypsin have shown that the enzyme hydrolyzes the amide bond of acylamino acid amides having general formula R′CO-NH (CHR) CO-NH_2. In most of the substrates tested, the group R′CO- was acetyl or benzoyl, admittedly unphysiological substituents. For example, chymotrypsin catalyzes the hydrolytic deamidation of acetyl-L-tyrosinamide and benzoyl-L-tyrosinamide (*1*). And only two acylamino acid amides in which the acyl substituents are glycyl and γ-L-glutamyl groups have been reported to be hydrolyzed by the action of chymotrypsin (*2*, *3*). Because of the increasing interest in the use of chymotrypsin in studies of protein structure, it seemed desirable to extensively the influence of various adjacent amino acid substituents to a normal sensitive amino acid residue on the susceptibility of the substrate to chymotrypsin.

In the present study, the aminoacyl-L-tyrosinamides in which the aminoacyl substituents are the aliphatic amino acid groups were prepared, and were subjected to the action of chymotrypsin. An experiment to test the rate of the susceptibility to chymotrypsin of other aminoacyl-L-tyrosine amides and ethyl esters has been in progress.

EXPERIMENTAL

Enzyme and Methods

α-Chymotrypsin was salt free, crystalline sample (CD 550–53) from Worthington Biochemical Corp., N. J., U. S. A., N per cent of it being found to be 14.66 by Dumas method. Enzyme solution was made up freshly in each experiment by weighing out the exact amount of solid, and dissolving it in 0.0025 *N* HCl. Alternatively, enzyme N concentrations were determined by measurements in Shimadzu spectrophotometer (QB-50) from a standard calibration curve at 280 mμ, which was established by enzyme N analysis. The purity of enzyme was established by its activity towards glycyl-L-tyrosinamide. At 25° and pH 7.8 (0.1 *M* phosphate buffer), the enzyme hydrolyzed 0.05 *M* glycyl-L-tyrosinamide

with a proteolytic coefficient ($C=k/e$)* of 0.0141. This value is significantly higher than that obtained under similar experimental conditions, *i.e.* $C=0.0065$ (*2*) and 0.0097 (*4*).

Unless otherwise stated, assays of enzyme activity were performed by the following procedure. An aliquot of enzyme solution was added to a 2 to 10 ml. assay flask containing a substrate in a specified concentration buffered with 0.01～0.05 *N* NaOH and 0.4 *M* phosphate at pH 8.0. Solution was made up to a specified volume by water, the final concentration of phosphate buffer being 0.1 *M*. It was incubated at 30.0°, and samples were withdrawn at various intervals for analysis. Hydrolysis rates were measured by titration of 0.2 to 1.0 ml. samples with 0.005 *N* HCl by means of the Conway microdiffusion method (*5*).

Synthesis of Peptide Derivatives

In general, the compounds tested as substrates were prepared as follows. The coupling reaction by the method of Vaughan and Osato (*6*) was performed by first preparing the requisite mixed anhydride at 0～−5° from the carbobenzoxy amino acid (0.02 mole) and isobutylchlorocarbonate (0.02 mole) in the presence of triethylamine (0.02 mole) and toluene (40 ml.). After 15 minutes, a mixture of the amino acid ethyl ester hydrochloride (0.02 mole), triethylamine (0.02 mole), and chloroform (40 ml.) was added. The reaction mixture was left at room temperature overnight, washed with water, 4 per cent bicarbonate solution, 2 per cent HCl, and water; the organic layer was dried over Na_2SO_4, and concentrated *in vacuo* to yield the desired carbobenzoxy dipeptide ester. For conversion of the ester to the corresponding amide, the compound (about 0.01 mole) was dissolved in 25～35 ml. of methanol previously saturated with dry NH_3 at 0°, and the solution was kept at room temperature for 2 days. The solution was then concentrated *in vacuo* to give carbobenzoxy dipeptide amide. The amide obtained was recrystallized from hot methanol-ether. For the removal of the carbobenzoxy group, the compound 0.003 mole) suspended in a mixture of methanol (9 ml.) and 0.5 *N* methanolic HCl (6.6 ml.) was treated with dry hydrogen in the presence of palladium black. The suspended crystals were dissolved gradually during the hydrogenation. The dipeptide amide hydrochloride thus obtained was recrystallized from methanol-ethyl acetate or methanol-ether.

Glycyl-L-*Tyrosinamide Hydrochloride* (*Gly*-L-*Tyr Am HCl*)—This compound was prepared by hydrogenolysis of 9.3 g. of the carbobenzoxy derivative (*7*). Yield, 6.5 g.; m.p. 217–218°; $[\alpha]_D^{19}$ +40.7° (c 2, in water).

$C_{11}H_{16}O_3N_3Cl$ (273.7)	Calcd.	N 15.4
	Found	N 15.6

The acetate of this dipeptide amide has been described previously (*7*).

L-*Alanyl*-L-*Tyrosinamide Hydrochloride* (L-*Ala*-L-*Tyr Am HCl*)—This compound was prepared from the carbobenzoxy derivative (*8*). Yield, 91 per cent; m.p. 236–237°; $[\alpha]_D^{20}$ +38.8° (c 2, in water).

$C_{12}H_{18}O_3N_3Cl$ (287.8)	Calcd.	C 50.1, H 6.3, N 14.6
	Found	C 49.9, H 6.1, N 15.0

The acetate of this amide has been described previously (*8*).

Carbobenzoxy-D-*Alanyl*-L-*Tyrosinamide*—This compound was prepared from carbobenzoxy-D-alanyl-L-tyrosine ethyl ester (*9*). Yield, 70 per cent; m.p. 175–176°; $[\alpha]_D^{12}$ 0° (c 2, in

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* k denotes the first order reaction constant as defined in Bergmann's work, *i.e.* $k=(1/t)\log[100/(100-\%\text{ hydrolysis})]$. t is in minute, e the protein concentration in mg. of protein N per ml. of assay solution.

DMF)*.

$C_{20}H_{23}O_5N_3$ (385.4) Calcd. N 10.9
Found N 10.8

D-*Alanyl*-L-*Tyrosinamide Hydrochloride* (D-*Ala*-L-*TyrAm HCl*)—This compound was prepared from the above amide and crystallized with difficulty. Yield, 95 per cent; $[\alpha]_D^{12}+16.2°$ (c 2, in water). The analysis was not satisfactory.

$C_{12}H_{18}O_3N_3Cl \cdot H_2O$ (305.8) Calcd. C 47.2, H 6.6, N 13.8
Found C 48.0, H 6.9, N 13.3

Carbobenzoxy-β-Alanyl-L-*Tyrosinamide*—This compound was prepared from carbobenzoxy-β-alanyl-L-tyrosine ethyl ester (*10*). Yield, 78 per cent; m.p. 199–200°; $[\alpha]_D^{14}-4.4°$ (c 2, in DMF).

$C_{20}H_{23}O_5N_3$ (385.4) Calcd. N 10.9
Found N 10.8

β-Alanyl-L-*Tyrosinamide Hydrochloride* (*β-Ala*-L-*TyrAm HCl*)—This compound was prepared from the above amide. Yield, 98 per cent; m.p. 230°; $[\alpha]_D^{20}+36.8°$ (c 2, in water).

$C_{12}H_{18}O_3N_3Cl$ (287.8) Calcd. C 50.1, H 6.3, N 14.6
Found C 49.9, H 6.4, N 14.8

Carbobenzoxy-L-*α-Amino-n-Butyroyl*-L-*Tyrosinamide*—The coupling reaction of carbobenzoxy-L-aminobutyric acid (*11*) and L-tyrosine ethyl ester gave an oily carbobenzoxy dipeptide ester, which was converted to the crystalline amide with an over-all yield of 52 per cent; m.p. 206–207°; $[\alpha]_D^{14}-15.3°$ (c 2, in DMF).

$C_{21}H_{25}O_5N_3$ (399.4) Calcd. N 10.4
Found N 10.4

L-*α-Amino-n-Butyroyl*-L-*Tyrosinamide Hydrochloride* (L-*But*-L-*TyrAm HCl*)—This compound was prepared from 0.71 g. of the above amide in a yield of 0.45 g.; m.p. 234–235°; $[\alpha]_D^{14}+52.8°$ (c 2, in water).

$C_{13}H_{20}O_3N_3Cl$ (301.8) Calcd. C 51.7, H 6.7, N 13.9
Found C 51.6, H 6.9, N 13.6

Carbobenzoxy-L-*Norvaline*—This compound was prepared in the usual manner from L-norvaline; yield, 79 per cent; m.p. 86°; $[\alpha]_D^{12}-4.2°$ (c 2, in acetone).

$C_{13}H_{17}O_4N$ (251.3) Calcd. N 5.6
Found N 5.6

Carbobenzoxy-L-*Norvalyl*-L-*Tyrosine Ethyl Ester*—This compound was prepared from the above compound and recrystallized from ethyl acetate-petroleum ether; yield, 86 per cent; m.p. 79–82°; $[\alpha]_D^{12}+14.5°$ (c 2, in glacial acetic acid).

$C_{24}H_{30}O_6N_2$ (442.5) Calcd. N 6.3
Found N 6.2

Carbobenzoxy-L-*Norvalyl*-L-*Tyrosinamide*—This compound was prepared from the above ester; yield, 78 per cent; m.p. 201°; $[\alpha]_D^{13}-20.6°$ (c 2, in DMF).

$C_{22}H_{27}O_5N_3$ (413.5) Calcd. N 10.2
Found N 10.2

L-*Norvalyl*-L-*Tyrosinamide Hydrochloride* (L-*Nval*-L-*TyrAm HCl*)—This compound was prepared from the above amide; yield, 91 per cent; m.p. 193–195°; $[\alpha]_D^{12}+43.3°$ (c 2, in water).

* Dimethylformamide is abbreviated in DMF.

$C_{14}H_{22}O_3N_3Cl$ (315.8) Calcd. N 13.3
Found N 13.3

Carbobenzoxy-L-*Valyl*-L-*Tyrosine Ethyl Ester*—This compound was prepared from carbobenzoxy-L-valine (*12*) in a yield of 77 per cent; m.p. 154°; $[\alpha]_D^{18}-2°$ (c 2, in glacial acetic acid).

$C_{24}H_{30}O_6N_2$ (442.5) Calcd. N 6.3
Found N 6.3

Carbobenzoxy-L-*Valyl*-L-*Tyrosinamide*—This compound was prepared from the above ester in a yield of 90 per cent; m.p. 199–200°; $[\alpha]_D^{14}-24.5°$ (c 2, in DMF).

$C_{22}H_{27}O_5N_3$ (413.5) Calcd. N 10.2
Found N 10.0

L-*Valyl*-L-*Tyrosinamide Hydrochloride* (L-*Val*-L-*TyrAm HCl*)—This compound was prepared from the above amide in a yield of 88 per cent; m.p. 167–174°; $[\alpha]_D^{20}+46.2°$ (c 2, in water).

$C_{14}H_{22}O_3N_3Cl$ (315.8) Cald. C 53.1, H 7.0, N 13.3
Found C 52.6, H 7.3, N 13.1

When this compound was dissolved in water and the equivalent amount of triethylamine was added, the beautiful crystals of free L-valyl-L-tyrosinamide appeared; m.p. 208°.

$C_{14}H_{21}O_3N_3$ (279.3) Calcd. N 15.0
Found N 15.1

Carbobenzoxy-L-*Norleucyl*-L-*Tyrosinamide*—The carbobenzoxy dipeptide ester was obtained from carbobenzoxy-L-norleucine (*11*) as an oil, and was converted to the amide. Over-all yield, 38 per cent; m.p. 202°; $[\alpha]_D^{14}-18.2°$ (c 2, in DMF).

$C_{23}H_{29}O_5N_3$ (427.5) Calcd. N 9.8
Found N 9.6

L-*Norleucyl*-L-*Tyrosinamide Hydrochloride* (L-*Nleu*-L-*TyrAm HCl*)—This was prepared from 0.73 g. of the above amide. Yield, 0.55 g.; m.p. 138–142°; $[\alpha]_D^{20}+33.4°$ (c 2, in water).

$C_{15}H_{24}O_3N_3Cl$ (329.8) Calcd. C 54.6, H 7.3, N 12.7
Found C 54.1, H 7.7, N 13.1

L-*Leucyl*-L-*Tyrosinamide Hydrochloride* (L-*Leu*-L-*TyrAm HCl*)—This compound was prepared from the oily carbobenzoxy derivative (*8*). Yield, 86 per cent; m.p. 128–133°; $[\alpha]_D^{20}+15.4°$ (c 2, in water).

$C_{15}H_{24}O_3N_3Cl$ (329.8) Calcd. C 54.6, H 7.3, N 12.7
Found C 54.2, H 7.6, N 12.3

The acetate of this amide has been described previously (*8*).

Carbobenzoxy-ε-*Amino-n-Caproyl*-L-*Tyrosine Ethyl Ester*—This compound was prepared from carbobenzoxy-ε-aminocaproic acid (*11*) in a yield of 82 per cent; m.p. 100–101°; $[\alpha]_D^{18}+23.0°$ (c 2, in glacial acetic acid)

$C_{25}H_{32}O_6N_2$ (456.5) Calcd. N 6.1
Found N 6.0

Carbobenzoxy-ε-*Amino-n-Caproyl*-L-*Tyrosinamide*—This compound was prepared from the above ester in a yield of 52 per cent; m.p. 159–160°; $[\alpha]_D^{14}+3.0°$ (c 2, in DMF).

$C_{23}H_{29}O_5N_3$ (427.5) Calcd. N 9.8
Found N 9.5

ε-*Amino-n-Caproyl*-L-*Tyrosinamide Hydrochloride* (ε-*Nleu*-L-*TyrAm HCl*)—This hygroscopic crystals were obtained from the above amide in a yield of 93 per cent; m.p. 129–133°;

$[\alpha]_D^{20}+26.0°$ (c 2, in water).

$C_{15}H_{24}O_3N_3Cl$ (329.8) Calcd. C 54.6, H 7.3, N 12.7
Found C 54.0, H 7.6, N 12.2

RESULTS AND DISCUSSION

pH-Activity Curve—Since an experiment concerning the optimum pH with an aminoacyl-L-tyrosinamide had not been reported, comparative measurements of the effect of pH on the amidase activity of chymotrypsin were made with Gly-L-TyrAm as a substrate, the results being shown in Fig. 1. As may be seen from Fig. 1, an optimum pH of the reaction appears

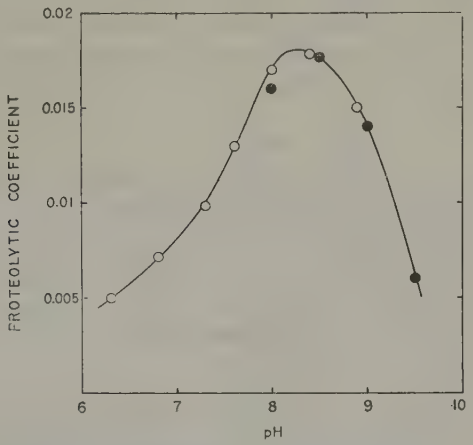

FIG. 1. The pH dependence of the hydrolysis of Gly-L-TyrAm (0.05 *M*) by chymotrypsin at 30°. —○—, 0.1 *M* phosphate buffer. —●—, 0.1 *M* with respect to the amine component of tris-(hydroxymethyl)-aminomethane-HCl buffer.

to be near 8.3. For the comparison of hydrolytic susceptibility of various aminoacyl-L-tyrosinamides, a phosphate buffer at pH 8.0 was used for fear that amino group of Tris might participate in the transamidation reaction.

It would be of interest to note that the optimum pH of acyl-L-tyrosinamides in which acyl groups are acetyl, benzoyl and nicotinyl (*4*, *13*, *14*) is reported to be at near 7.9, and this value is slightly lower than that of Gly-L-TyrAm.

Relation between Proteolytic Coefficients and Initial Substrate Concentrations—Bergmann and Fruton have used the proteolytic coefficient to compare the activity of an enzyme towards several structurally related substrates at a single, initial substrate concentration (*15*), while Neurath and coworkers showed that the plots of the proteolytic coefficients for the hydrolysis of two substrates by chymotrypsin *versus* initial substrate concentration yielded sometimes crossed curves, which resulted from varying ratios of these coefficients (*1*, *4*). Therefore, it seems desirable to compare the values of

proteolytic coefficients in various substrate concentrations and $C_{max.}$ which will be described in later section for the comparison of the sensibility to chymotrypsin of aminoacyl-L-tyrosinamides.

In this study, the glycyl residue of Gly-L-TyrAm was replaced by the following: L-alanyl, L-α-amino-n-butyroyl, L-norvalyl, L-norleucyl, D-alanyl, β-alanyl, L-valyl, L-leucyl, ε-aminocaproyl. Since the compounds with larger side chains such as n-butyl or isobutyl on the N-terminal residue were sparingly soluble in water at pH 8.0, they were not tested at such high substrate concentrations as 0.05 M or 0.025 M. However, all the substrates were tested at least at 0.01 M substrate concentration. Under the conditions given in the section of *Enzyme and Methods*, it was found that the hydrolysis of the substrates tested followed first order kinetics within the extent of error in all cases except L-Leu-L-TyrAm, and that proteolytic coefficients increased with decreasing initial substrate concentration. Representative data for the hydrolysis of L-Ala-L-TyrAm at two different substrate

TABLE I

Example in Measurements of Hydrolysis of L-Ala-L-TyrAm and L-Leu-L-TyrAm by Chymotrypsin

pH 8.0 (0.1 M phosphate buffer); temperature, 30°.

Substrate	Substrate concentration (M)	Enzyme concentration (*mg. protein* N *per ml.*)	Time (*min.*)	Hydrolysis (%)	Proteolytic coefficient, C
L-Ala-L-TyrAm	0.05	0.109	22.5	29.4	0.062
			38.7	46.0	0.064
			52.0	57.4	0.065
			69.5	68.0	0.065
			90.5	76.3	0.063
	0.01		14.3	23.8	0.076
			30.3	44.5	0.077
			43.3	56.2	0.076
			61.5	68.5	0.075
			84.5	80.3	0.077
L-Leu-L-TyrAm	0.01	0.0096	29.7	18.0	0.30
			48.0	25.9	0.28
			67.2	33.1	0.27
			87.7	36.8	0.24
			106	41.8	0.23
	0.0025		20.0	18.5	0.46
			33.7	28.0	0.44
			58.5	39.0	0.38
			72.6	42.3	0.34
			91.5	48.0	0.32

concentrations are shown in Table I as an example. A summary of all coefficients determined are given in Table II.

TABLE II

Proteolytic Coefficients of Aminoacyl-L-Tyrosinamides in Various Initial Substrate Concentrations

pH 8.0 (0.1 *M* phosphate buffer); temp., 30°.

Substrate	Proteolytic coefficient, *C*				
	0.05 *M*	0.025 *M*	0.01 *M*	0.005 *M*	0.0025 *M*
Gly-L-TyrAm	0.017	0.020	0.021		
L-Ala-L-TyrAm	0.064	0.072	0.076		
L-But-L-TyrAm	0.16	0.24	0.26		
L-Nval-L-TyrAm			0.44	0.49	0.51
L-Nleu-L-TyrAm			0.43	0.48	0.49
D-Ala-L-TyrAm	0.0031	0.0037	0.0046		
β-Ala-L-TyrAm	0.012	0.017	0.020		
L-Val-L-TyrAm		0.30	0.32	0.33	
L-Leu-L-TyrAm			0.33[a]	0.44[a]	0.50[a]
ε-Nleu-L-TyrAm		0.089	0.16	0.20	

a) The values given are extrapolated initial constants, since decreasing values of *C* were observed.

The hydrolysis of L-Leu-L-TyrAm does not follow the kinetics of first order as shown in Table I. It was observed that the apparent proteolytic coefficients of this substrate decreased linearly with increasing the time of hydrolysis as may be seen in Table I. The decreasing constants appear to be due to progressive inhibition by the liberated L-Leu-L-Tyr. As Kimmel and Smith suggested already in the case of papain on the synthetic substrates (*16*), the proteolytic coefficients at the zero time extraporated graphically, which are shown in Table II, were taken for the comparison of sensibility of the substrate.

Reaction Kinetics and $C_{max.}$—Kinetic constants were determined from the Michaelis-Menten formulation in which E is enzyme, and S substrate.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + \text{products}$$

The expression of the reaction under steady state conditions can be given in linear form (*17*);

$$[S]/V_i = [S]/V_{max.} + K_m/V_{max.}$$

where $K_m = (k_2 + k_3)/k_1$. Since, in the case of first order reaction kinetics, $[S]/V_i = 1/(2.3k)$, where $k = (1/t) \log [100/(100 - \%\ \text{hydrolysis})]$, a line with a slope of $1/V_{max.}$ and ordinate intercept of $K_m/V_{max.}$ is obtained, when $1/(2.3k)$ is plotted against $[S]$. k_3 is calculated by the equation $V_{max.} = k_3 e$, where e

is total concentration of enzyme in mg. of protein N per ml. The line fitting the plot of $[S]/V_i$ *versus* $[S]$ was calculated by a method of least squares. If first order reaction constants are extraporated to zero initial substrate concentrations, the corresponding maximum proteolytic coefficient is (*1*),

$$C_{\max.} = \frac{k_3}{2.3\ K_m}$$

A plot for the hydrolysis of L-Ala-L-TyrAm is shown in Fig. 2 as a representative example. Since the values of the first order reaction constant of L-Leu-L-TyrAm decrease progressively as stated before, extrapolated initial k value was used expediently for determination K_m and k_3 by which $C_{\max}$ was calculated. A summary of constants of the substrates are given in Table III.

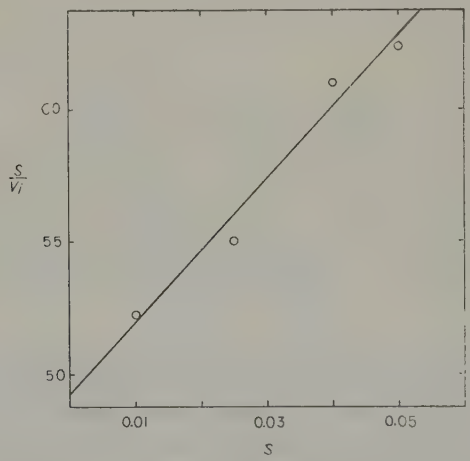

FIG. 2. A plot of initial substrate concentration $[S]$ divided by initial velocity (V_i) *versus* $[S]$ for the hydrolysis of L-Ala-L-TyrAm at 30°, pH 8.0, and 0.109 mg. protein N/ml. $K_m/V_{\max.}=49.2$; $1/V_{\max.}=1/(k_3\,e)=273$.

As shown in Table II and III, all the compounds having N-terminal straight chain aliphatic L-amino acid were hydrolyzed more rapidly than the corresponding glycyl compound. The introduction of the side chain of a larger L-amino acid residue causes a marked increase in the rate, and it appears that, although further experiments with the substrate having N-terminal C_7 or C_8 straight chain aliphatic amino acid in aminoacyl-L-tyrosinamide will be required for the definite conclusion, a maximum value of the proteolytic coefficient is shown for L-Nval-L-TyrAm or L-Nleu-L-TyrAm.

It is of interest to note that the appearance of the increase in the rate of hydrolysis by chymotrypsin of aminoacyl-L-tyrosinamides is similar to that of L-amino acid amides by leucine aminopeptidase (*18*). A further point of interest is that the replacements of the glycyl residue of Gly-L-TyrAm

by larger L-amino acid residues lead to a significant decrease in $C_{max.}$ of the substrates to Cathepsin C which has a substrate specificity similar to that of chymotrypsin (*9*, *19*).

TABLE III

Kinetic Constants of Aminoacyl-L-Tyrosinamides

pH 8.0 (0.1 *M* phosphate buffer); temp., 30°.

Substrate	K_m (*M*)	k_3[a)]	$C_{max.}$
Gly-L-TyrAm	0.15	0.0078	0.023
L-Ala-L-TyrAm	0.18	0.034	0.082
L-But-L-TyrAm	0.050	0.038	0.33
L-Nval-L-TyrAm	0.051	0.062	0.53
L-Nleu-L-TyrAm	0.043	0.051	0.52
D-Ala-L-TyrAm	0.072	0.00085	0.0051
β-Ala-L-TyrAm	0.047	0.0027	0.025
L-Val-L-TyrAm	0.23	0.18	0.34
L-Leu-L-TyrAm	0.012	0.017	0.62
ε-Nleu-L-TyrAm	0.011	0.0074	0.29

a) In *M*/liter/minute/mg. protein N/ml.

It would be of interest to note that the D-alanyl compound is hydrolyzed at about 1/16 times the rate for L-Ala-L-TyrAm as shown in Table I and III, because a similar ratio of 1 to 19 has been found for the hydrolysis by Cathepsin C of D-Ala-L-TyrEt and L-Ala-L-TyrEt (*19*), and 1 to 27 for that by leucine aminopeptidase of L-Leu-D-Ala and L-Leu-L-Ala (*18*). In this connection, an excellent discussion concerning the optical specificity to carboxypeptidase of the substrates given by Yanari and Mitz (*20*) would be suggestive.

The substitution of the glycyl residue of Gly-L-TyrAm by β-alanyl residue leads to a slight increase in $C_{max.}$ (Table III), while the decrease in the proteolytic coefficients at high substrate concentrations (Table I). This is another example that the crossing of the curves, obtained when the proteolytic coefficients for the hydrolysis of two substrates are plotted against initial substrate concentrations, is observed. It may be noteworthy that Cathepsin C shows no detectable action on β-Ala-L-TyrEt (*10*) and chymotrypsin hydrolyzes γ-L-Glu-L-PheAm at about 1/8 times the rate for Gly-L-PheAm (*3*).

Paperchromatography of Reaction Mixture—It has been recognized that, in addition to the catalysis of hydrolytic reaction, chymotrypsin also catalyzes transpeptidation reaction (*21*). In this study, the possibility of the occurrence of transpeptidation reaction was tested by means of paperchromatography. The R_f values of the reference compounds are given in Table IV.

In addition to measurements by Conway method of the amount of ammonia liberated, the reactions were followed by transferring 5–20 μl.

samples at various times on filter papers. In all cases, rapid hydrolysis of the substrate occurred yielding free aminoacyltyrosine peptide, although additional faint spot of an unkonwn, new substance was revealed in the cases of L-Ala-L-TyrAm, L-But-L-TyrAm, L-Nleu-L-TyrAm and L-Leu-L-TyrAm, the R_f values of unknown substance being shown in Table IV.

TABLE IV

R_f Values of Reference Compounds[a]

The compounds were chromatographed on Toyo Roshi No. 50 Paper.

Substance	*n*-Butanol : acetic acid : pyridine : water	
	30:6:20:24, by vol.	4:1:1:2, by vol.
Gly-L-TyrAm	0.39	0.37
Gly-L-Tyr	0.33	0.32
L-Ala-L-TyrAm	0.50	0.44
L-Ala-L-Tyr	0.43	0.39
L-But-L-TyrAm	0.56	0.54
L-But-L-Tyr	0.50	0.51
L-Nval-L-TyrAm	0.68	0.66
L-Nval-L-Tyr	0.62	0.64
L-Nleu-L-TyrAm	0.76	0.70
L-Nleu-L-Tyr	0.72	0.68
β-Ala-L-TyrAm	0.40	0.36
β-Ala-L-Tyr	0.31	0.32
L-Val-L-TyrAm	0.66	0.61
L-Val-L-Tyr	0.60	0.58
L-Leu-L-TyrAm	0.75	0.72
L-Leu-L-Tyr	0.69	0.67
ε-Nleu-L-TyrAm	0.48	0.43
ε-Nleu-L-Tyr	0.41	0.39
New spot from digest of		
L-Ala-L-TyrAm	0.53	0.51
„ L-But-L-TyrAm	0.68	0.62
„ L-Nleu-L-TyrAm	0.55	
„ L-Leu-L-TyrAm	0.54	

a) The synthesis of aminoacyl-L-tyrosines has been described in previous communication from this laboratory (*11*). The synthesis of L-Nval-L-Tyr has not been published yet.

A representative chromatogram with L-Ala-L-TyrAm is given in Fig. 3.

The reaction mixture from L-Ala-L-TyrAm showed the most distinct spot of the new substance than other three substrates, but even in this case, the amount of it, compared with L-Ala-L-Tyr liberated, was assumed at 0.5 per cent or less qualitatively on the basis that the sensibilities to ninhydrin

of these two substance are about same extent. It may be taken that the proteolytic coefficients and kinetic constants of the substrates tested represent significantly the result of the direct hydrolysis by chymotrypsin, as the new substances described above are negligibly small in their amount even if they are due to transpeptidation.

It has been reported in this connection that α-glycyl-L-lysinamide is solely hydrolyzed by trypsin, occurring no tanspeptidation reaction (*22*).

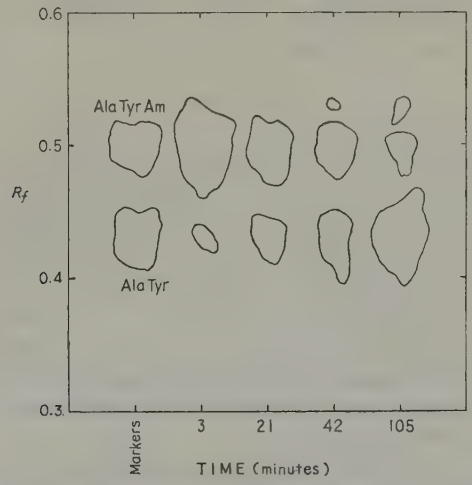

FIG. 3. Chromatogram showing the action of chymotrypsin on L-Ala-L-TyrAm at 30°, pH 8.0, 0.109 mg. protein N/ml. and 0.05 *M* of initial substrate concentration. The samples (approx. 8 μl.) were transfered to the starting line of a chromatogram; a drop of 2 *N* acetic acid had previously been applied to this position. Solvent system: *n*-butanol : acetic acid : pyridine : water (30 : 6 : 20 : 24, by vol.). A solution of ninhydrin (0.2 per cent) in isopropanol was used for the development of a chromatogram.

SUMMARY

1. A number of aminoacyl-L-tyrosinamide hydrochlorides in which the aminoacyl substituents are the aliphatic amino acid groups have been synthesized and tested as substrates for α-chymotrypsin.

2. By the use of glycyl-L-tyrosinamide, the pH optimum of chymotryptic hydrolysis was found to be near 8.3.

3. The values of proteolytic coefficients in various initial substrate concentrations and $C_{max.}$ were estimated, and were taken as measures of the relative susceptibility of hydrolysis of the substrates by chymotrypsin.

4. The rate of action of chymotrypsin was increased markedly by the presence of large side chain groups in the N-terminal L-amino acid residue of the substrate.

5. The presence of D-alanyl residue in the substrate rendered the sensitive

bond more resistant to hydrolysis by chymotrypsin.

6. Chromatographic analysis of the incubation mixture proved that although L-alanyl-, L-aminobutyroyl-, L-norleucyl- and L-leucyl-L-tyrosinamide produce an unknown substance positive for ninhydrin in very slight extent, all the substrates are hydrolyzed significantly by chymotrypsin.

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STUDIES ON RIBONUCLEASES IN TAKADIASTASE

II. SPECIFICITY OF RIBONUCLEASE T_1*

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In the previous paper (*2*) it was reported that in Takadiastase there are at least two thermostable RNases**, RNase T_1 and RNase T_2. RNase T_1 was obtained in almost homogeneous state and free from RNase T_2. The specificity of RNase T_1 was quite different from that of pancreatic RNase (RNase I) and other ribonucleodepolymerases, namely, it was only guanylic acid that was detected as mononucleotide in the digestion product of yeast RNA. In order to further clarify the specificity of RNase T_1, identification of mononucleotides and terminal groups of oligonucleotides produced by the enzyme from yeast RNA was carried out.

EXPERIMENTAL

Preparation of Enzymes

RNase T_1—RNase T_1 used in the present experiment was prepared as described in the previous paper and zone electrophoretically almost homogeneous. Its specific activity was 370-fold of that of crude extract.

Phosphomonoesterase—PMase used was prepared by the method of Schmidt (*3*) from prostate. At first the thawed glands were cut into small pieces, homogenized and extracted with 0.9 per cent NaCl. After autolysis with 2–3 drops of toluene overnight, dialysis, and adjusting pH to 5.5 with acetic acid, the precipitates formed were successively centrifuged off. Then the PMase was precipitated with ammonium sulfate. Activity of PMase in the dialyzed solution was about 300 units/ml.

RNA Digestion by RNase T_1

In all the experiment in this report, reaction mixture contains 10 mg. of commercial yeast RNA (Schwarz), 10 μg. of RNase T_1; 0.2 *M* Tris buffer, 0.1 ml., pH 7.5; EDTA (10^{-3} *M* in final concentration) and total volume was 0.5 ml. Aliquots of the reaction

* Aided by a grant from the Scientific Research Fund of the Ministry of Education. A part of this work was presented at the meeting of "Société franco-japonaise de biologie" at Nagoya in August, 1957 (*1*).

** Abbreviations: ribonuclease, RNase; ribonucleic acid, RNA; phosphomonoesterase, PMase; guanosine-2′, 3′-cyclic phosphate, Gp!; 2′-guanylic acid, 2′-Gp; 3′-guanylic acid, 3′-Gp; 2′-adenylic acid, 2′-Ap; 3′-adenylic acid, 3′-Ap; cytidylic acid, Cp; uridylic acid, Up.

mixture were taken at intervals, diluted to a concentration of 3 mg. RNA/ml., and equal volume of uranium reagent (0.25 per cent uranyl acetate in 2.5 per cent trichloroacetic acid) was added to determine the uranium reagent soluble organic phosphate.

Paper Chromatography

Solvents used for the detection of Gp! were; isopropanol : water (7 : 3) with ammonia in the vapour phase (solvent I) (*4*) and isopropanol : saturated ammonium sulfate : water (2 : 79 : 19) (solvent II) (*5*). To separate nucleosides and nucleotides solvent I and solvent III (butanol : water : ammonium hydroxide, (86 : 14 : 5)) (*6*) were used. Paper used was Tôyôroshi No. 51 and the methods employed to detect the spots were those described in previous paper. For the purpose of quantitative measurement each spot was cut off, eluted with 0.1 *N* HCl and the optical density was measured.

RESULTS

Guanosine-2′, 3′-Cyclic Phosphate as an Intermediary Product—As described in previous paper (*2*), when digestion mixture of yeast RNA was at various time intervals chromatographed with solvent II, an unknown spot of R_f between 3′- and 2′-adenylic acid appeared at earlier stages of digestion and next appeared the spot of 3′-Gp with simultaneous fading of the unknown spot (Fig. 1-a). To identify the unknown spot 1/10 volume of *N* HCl was added to the reaction mixture of each interval and kept overnight at 2° (under the acidic condition uranium reagent soluble phosphate was not increased), then chromatographed with the same solvent. As shown in Fig. 1-b, by the acid treatment the unknown spot faded* and in place of it increase in 3′-Gp and appearance of 2′-Gp were observed.

The mixture of the 15 hours digestion by RNase T_1 was incubated at 37° for 2 hours with PMase before and after the acid treatment and inorganic phosphate liberated was measured. In the former case only 15 per cent of total phosphate was liberated as inorganic phosphate, while in the latter 22 per cent of total phosphate. So phosphate group of the compound which gave the unknown spot is not in the form of monoester and can be changed to a monoester by HCl treatment.

On the other hand, when the dialysate of digestion mixture was chromatographed with solvent I, one of the main spots was found to correspond to those of Gp! (*7*). The spot was eluted and identified as Gp! by the following methods: (*a*) acid hydrolysis, (*b*) alkaline hydrolysis, (*c*) RNase T_1 digestion, (*d*) acid hydrolysis followed by incubation with PMase and alkaline hydrolysis (Fig. 2) and (*e*) measurement of ultraviolet absorption spectrum. By these treatments, Gp was produced in (*a*), (*b*), (*c*), and guanosine in (*d*). Besides the results it was proved by using solvent II that enzymic hydrolysis produced 3′-Gp, acid and alkaline hydrolysis produced both 2′-

* The unknown spot had a fluorescence which was characteristic of guanine derivatives. But after acid treatment the faint spots remained on the position of the unknown spot had no more fluorescence, so these spots may be some di- or trinucleotides which exist also in the RNase I resistant fraction (*2*).

and 3′-Gp and the fourth treatment gave only guanosine. So it is clear that RNase T_1 produces Gp! as intermediary product of digestion.

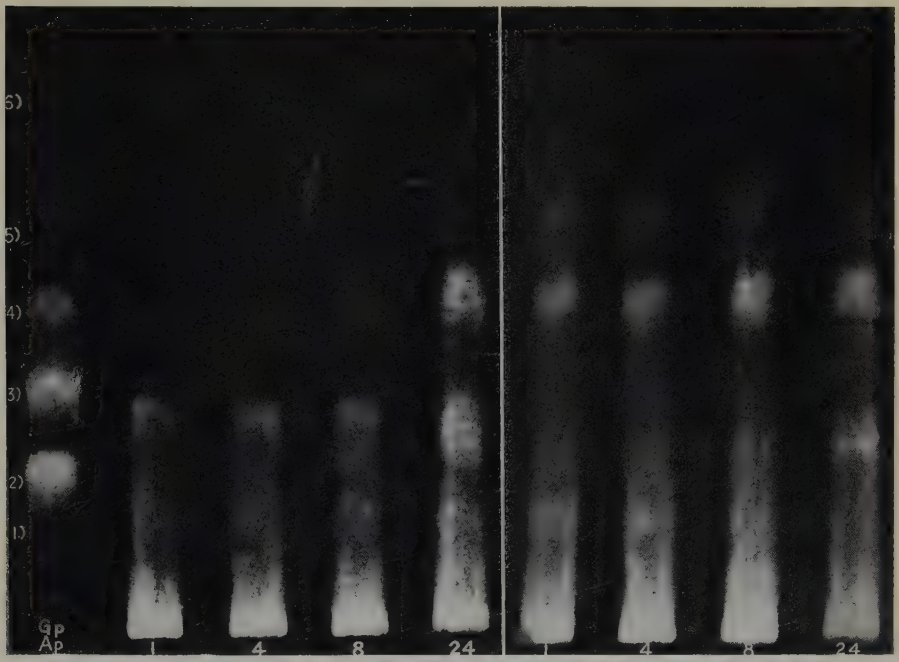


FIG. 1. Paper chromatograms of RNase T_1 digestion of yeast RNA. (a) Time course of the digestion. The reaction mixtures as shown in EXPERIMENTAL were incubated at 37° for 1, 4, 8 and 24 hours respectively. The spots are: (1), certain di- or trinucleotides; (2), 3′-Ap; (3), 2′-Ap; (4). 3′-Gp; (5). 2′-Gp; 6), Cp and Up. Solvent II was used. (b) After the incubation indicated in the (a) 1/10 volume of *N* HCl was added to the each reaction mixture and kept overnight at 2°.

Limited Digestion of Yeast RNA by RNase T_1—On RNA digestion by RNase T_1 uranium reagent soluble organic phosphate reached maximum in amount in 4 hours, about 27 per cent of total phosphate. Inorganic phosphate after incubation of digestion mixture with PMase also reached maximum in 4 hours, about 22 per cent of total phosphate. Monoguanylic acid (Gp!+3′-Gp) produced was about 40 per cent of total guanylic acid content. Other mononucleotides were scarcely liberated by 15 hours digestion. A fraction remains undecomposed under the condition described above. It may be called "RNase T_1 resistant fraction".

Determination of Terminal Nucleotides—In order to further clarify the specificity of the enzyme, mononucleotides and terminal groups of oligonucleotides produced by RNase T_1 from yeast RNA were identified by the modified method of Volkin and Cohn (*8*). The digestion mixture was kept overnight in 0.1 *N* HCl at 2° to split only the cyclic phosphate to 2′-, or 3′-phosphate and inactivate RNase T_1 irreversibly, and after neutralization

FIG. 2. Identification of guanosine-2′, 3′-cyclic phosphate. Solvent I was used. Spots are: I, authentic samples of Gp and Ap; II, the product which appeared initially on digestion of RNase T_1, the R_f value corresponding to that of Gp!; III, digestion product of spot II by RNase T_1; IV, acid hydrolysis product of spot II (0.1 *N* HCl at 37° for 2 hours); V, alkaline hydrolysis product of spot II (*N* NaOH at 37° for 24 hours); VI, spot II treated successively by three procedures, acid hydrolysis, PMase digestion and alkaline hydrolysis, R_f value of this spot corresponds to that of guanosine. White broken circles indicate the position of faint spots.

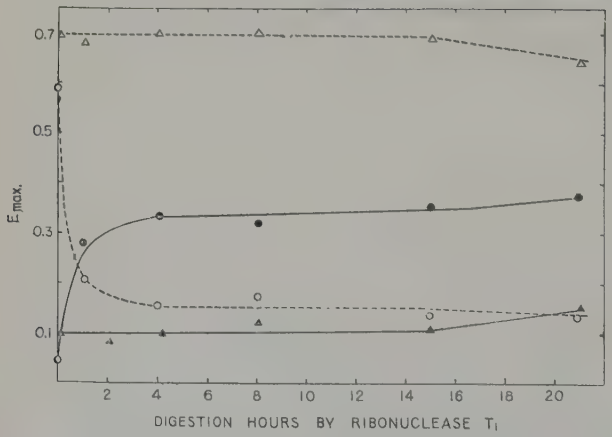


FIG. 3. Terminal nucleosides and internal nucleotides produced by incubation with RNase T_1 followed by the splitting of terminal phosphate group by PMase and alkaline hydrolysis. Initial reaction mixture is the same as in EXPERIMENTAL. PMase treatment was carried out for 2 hours with about 40 units of enzyme at 37° at pH 5.5. Aliquots of the alkaline hydrolysate were chromatographed on paper after neutralization using the solvent I and each spot was eluted by 0.1 *N* HCl for 48 hours. The optical density at maximum absorption of each spot was measured. —●—, guanosine; —▲—, other nucleosides; --○--, guanylic acid; --△--, other nucleotides.

it was incubated with PMase. By this incubation the terminal phosphate group produced by RNase T_1 digestion was splitted off and inorganic phosphate was liberated. Then alkaline hydrolysis was carried out in *N* NaOH at 37° for 24 hours. Nucleosides produced from the terminal nucleotides and mononucleotides arised from internal nucleotides were chromatographically separated on paper using the solvent I. The spots of nucleotide and nucleoside were cut off, eluted and optical density of their maxium absorption was measured. The result was that the terminal nucleoside produced after alkaline hydrolysis was only guanosine till 15 hours digestion by RNase T_1, whereas other nucleosides remained at the amount to be produced from the terminals of original RNA. Corresponding with the results the amount of guanylic acid combined within the chain and not dephosphorylated by PMase rapidly decreased in initial stages and reached minimum in 4 hours, while other nucleotides were almost constant till 15 hours (Fig. 3).

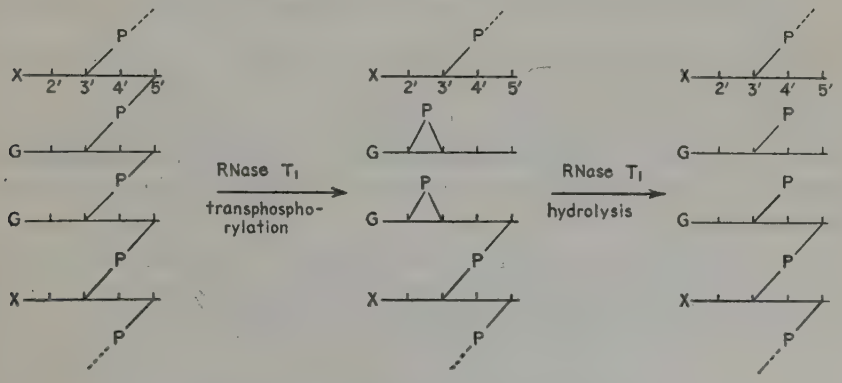
The amounts of gnanylic acid were apparently high comparing with the amount calculated with inorganic phosphate produced from the terminal phosphate. It seems to be owing to imcomplete separation from other nucleotides which move forward in the paper chromatography.

DISCUSSION

From the results described above it is clear that RNase T_1 splits the secondary phosphate ester bonds of guanosine-3′-phosphate through the guanosine-2′,3′-cyclic phosphate as the intermediate. The mechanism of RNA digestion by RNAase T_1 is divided into two steps transphosphorylation and hydrolysis, as in the case of RNase I (*9*, *10*).

SCHEME

Mechanism of RNA digestion by RNase T_1



So far experiments were carried out with the amount of RNase T_1 from 1 μg./0.5 ml. to 100 μg./0.5 ml. the degree of digestion and the amount of terminal nucleotides remained unchanged.

However, when the RNase T_1 resistant fraction remained after digestion under the condition described above was reincubated with much larger amount of the enzyme (1 mg./0.5 ml.), it was slowly further degraded. It has not yet been clarified whether RNase T_1 itself had the slight activity to split other phosphodiester bonds or trace amount of some other contaminant RNases revealed the activity.

At any rate the specificity of RNase T_1 agrees with the results described in the previous report (*2*), that RNase T_1 degraded the enzymically synthesized RNA (*11*) as well as natural yeast RNA but did not split the polyadenylic acid synthesized similarly, and that cyclic adenylic acid and cytidylic acid were scarcely hydrolysed.

It is very interesting to compare it with specificities of other ribonucleodepolymerases, for example, RNase I which splits the secondary phosphate ester bonds of pyrimidine nucleoside-3′-phosphate. It is first time that RNase which has only one nuclotide specificity, namely, splitting the secondary phosphate ester bond concerning the guanosine-3′-phosphate. It is useful for studing the chemical structure of RNA and oligonucleotides as RNase I and others. The fact that appreciable amount of guanylic acid was liberated as mononucleotide by the action of RNase T_1, seems to indicate that in the yeast RNA the structure in which two or more guanylic acid residues link adjacently may exist considerably.

SUMMARY

1. Guanosine-2′,3′-cyclic phosphate and 3′-guanylic acid are formed as the digestion products of yeast RNA by RNase T_1 at the initial and at the later stage respectively.

2. RNA digestion by RNase T_1 is incomplete, and a resistant fraction remains.

3. Mononucleotide and the terminal nucleotides of oligonucleotides produced by RNase T_1 are exclusively guanylic acid.

4. Consequently it is clear that RNase T_1 splits the secondary phosphate ester bound of guanosine-3′-phosphate involving the formation of guanosine-2′,3′-cyclic phosphate as an intermediate and producing guanosine-3′-phosphate.

The auther wishes to thank Prof. F. Egami for his helpful discussion and encouragement. She is indebted to Sankyo Co. Ltd. for the gift of "Takadiastase Sankyo", and to Dr. E. Iwase for the gift of U.V. filter of Scientific Research Institute.

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The Journal of Biochemistry, Vol. 45, No. 1, 1959

STUDIES ON THE METABOLISM OF LEUCONOSTOC MESENTEROIDES P-60

II. 8-AZAGUANINE RESISTANT- AND 8-AZAXANTHINE RESISTANT STRAINS

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(Received for publication, April 7, 1958)

In the previous paper, it was reported that the growth of *Leuconostoc mesenteroides* P-60 was remarkably inhibited by 8-azaguanine or 8-azaxanthine. There have been many reports concerning strains resistant to the purine-antimetabolites (*1-6*), but little is known about 8-azapurine resistant micro-organisms. After successive generations of *L. mesenteroides* culture, during which the concentration of antimetabolite was gradually increased, 8-azaguanine resistant- and 8-azaxanthine resistant-strains of *L. mesenteroides* P-60 were successfully obtained. Some studies were made of their morphological characteristics and biochemical behaviours. In the present paper, several biochemical changes in the purine metabolism of the resistant strains are reported.

EXPERIMENTALS AND RESULTS

Experimental methods were the same as described in the previous paper (*8*).

1. Isolation of the Resistant-Strains—Successive cultivations in basal medium supplemented with hypoxanthine (10 μg./ml.) and increasing amounts of 8-azaguanine produced 8-azaguanine resistant *L. mesenteroides* P-60. After about ten generations (30 days), an 8-azaguanine resistant strain (AGR strain) was able to grow even in the medium saturated with 8-azaguanine at a concentration of 500 μg. per ml.

An 8-azaxanthine resistant strain (AXR strain) of the same degree of resistance was obtained in the same manner.

2. Purine Requirements—The rates of growth of both resistant strains in the various media were compared with that of the original strain (0 strain) (Fig. 1). The characteristic purine requirement of 0 strain was not evident in both the AGR and AXR strains. The latter could even grow in the non-supplemented basal medium and the addition of purines did not produced any growth promoting effect.

3. Growth-Inhibiting Effect of Purine-Antimetabolites—The growth-inhibiting effect of purine-antimetabolites on both strains was compared with its effect

on 0 strain (Fig. 2). Among the seven antimetabolites examined, 8-azahypoxanthine, 8-azaadenine and 2,6-diaminopurine had no effect on AGR and and AXR strains as well as on 0 strain at a concentration lower than 500 μg./ml. Aminopterin and 6-mercaptopurine which had some inhibitory effects on 0 strain were found to have no effect on both resistant strains. The

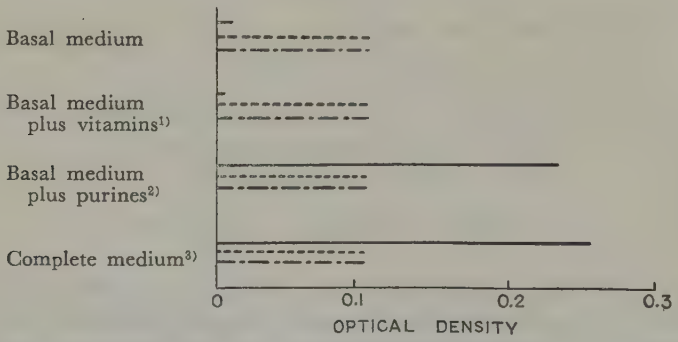

FIG. 1. Growth of 3 strains in various media (24 hours). ——: Original strain, - - - -: AGR strain, —·—·—: AXR strain.

1. PABA 1 μg./ml., pteroylglutamic acid 0.01 μg./ml. and vitamine B_{12} 0.01 μg./ml.,
2. Hypoxanthine, xanthine, guanine, adenine: each 10 μg./ml.
3. Basal medium plus vitamins, purines and uracil.

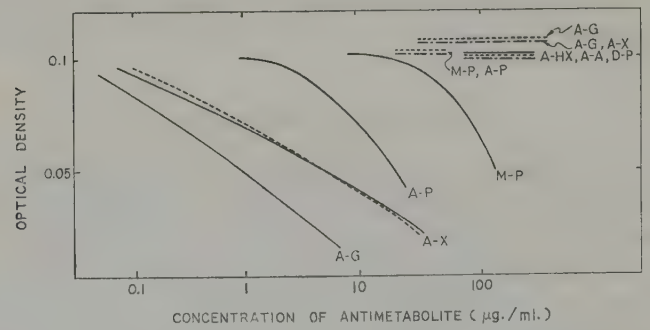

FIG 2. Effect of antimetabolites to 3 strains. ——: Original strain, - - - -: AGR strain, —·—·—: AXR strain.

A-G: 8-Azaguanine, A-X: 8-Azaxanthine, A-HX: 8-Azahypoxanthine, A-A: 8-Azaadenine, D-P: 2,6-Diaminopurine, M-P: 6-Mercaptopurine, A-P: Aminopterine.

Medium: O-Strain—Basal medium plus hypoxanthine (10 μg./ml.).
AGR and AXR Strain—Basal medium.

susceptibility of AGR and AXR strains to 8-azaguanine and 8-azaxanthine was of remarkable difference. The growth of AGR strain was inhibited by 8-azaxanthine, as much as on the 0 strain, but the growth of AXR strain was not inhibited at all by 8-azaguanine and 8-azazanthine.

DISCUSSION

The fact that AGR and AXR strains could grow in the purine-free basal medium revealed that a remarkable metabolic alteration occurred in these strains (*Exp. 2*, Fig. 1). It could be postulated that the resistant strains might have acquired the ability to biosynthesize essential purines. L a w (*7*) reported that the cells of 8-azaguanine resistant Leukemia became sensitive to pteroyl glutamic acid antimetabolite, suggesting a change of the enzyme system involved in purine biosynthesis. The fact that the AGR and AXR strains showed diminished sensitivity to 6-mercaptopurine and aminopterin (*Exp. 3*, Fig. 2) would suggest that these strains were able to synthesize purine without being inhibited by these compounds. In addition, AGR strain is metabolically different from AXR strain since AGR strain, like the 0 strain, is sensitive to 8-azaxanthine (*Exp. 3*, Fig. 2). In another experiment the authors found that the inhibitory effect of 8-azaxanthine on the growth of AGR strain was reduced by some of the natural purines, and that the competitive effect of xanthine was the strongest and that of hypoxanthine was next and that of guanine was the weakest. These findings should throw some light on the schematic pathways of purine intermediary metabolism.

SUMMARY

The purine metabolism of the 8-azaguanine resistant and 8-azaxanthine resistant strains of *Leuconostoc mesenteroides* P-60 were compared with that of the original strain. These resistant strains did not require preformed exogenous purines for their growth and were less sensitive to aminopterin and 6-mercaptopurine than the original strain. While the growth of the azaguanine-resistant strain was inhibited by 8-azaxanthine like the original strain, azaxanthine-resistant strain was not inhibited by 8-azaguanine.

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The Journal of Biochemistry, Vol. 46, No. 1, 1959

STUDIES ON CYTOCHROME A

II. SPECTRAL PROPERTIES OF CYTOCHROME A*

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(Received for publication, April 7, 1958)

Keilin and Hartree (*1*) were the assumption that cytochrome a preparation contained an a_3 component identical with the respiration enzyme. Since then many observations (*2–7*) have apparently supported this opinion and it has been concluded that the difference between cytochromes a and a_3 is in the behavior towards compounds such as carbon monoxide and cyanide, to which cytochrome a is inert. However, based on the observations that their cytochrome a preparation had no cytochrome oxidase activity, but could combine with carbon monoxide and cyanide, a different interpretation of the spectrum of cytochrome a was given by Yakushiji and Okunuki (*8*, *9*). Thus, to elucidate the relation between cytochrome a and cytochrome oxidase, studies on the behavior of cytochrome a towards carbon monoxide and cyanide are extremely important. The present paper gives results of a spectral analysis of the effect of carbon monoxide, cyanide, nitric oxide, and oxygen on our cytochrome a preparation (*11*). Evidences are presented that cytochrome a can react with these compounds.

EXPERIMENTAL

Cytochrome a was prepared by the method described in the previous paper (*11*). For spectrophotometric measurements the preparation was diluted to a suitable concentration with 0.1 *M* phosphate buffer containing 1 per cent sodium cholate (pH 7.4). Carbon monoxide generated by dropping formic acid into concentrated sulfuric acid at 140–160°, was washed with saturated potassium permanganate and then with 50 per cent potassium hydroxide solution. Nitric oxide was generated by dropping a mixture of 1 *M* sodium nitrite and 1 *M* potassium ferrocyanide into a solution consisting of 1 part of glacial acetic acid and 2 parts of water. It was purified by passage through 50 per cent potassium hydroxide and concentrated sulfuric acid. All gases used were passed through the cytochrome a solution for 10–15 minutes. Spectrophotometric measurements were made in a closed Thunberg tube type cell. Other materials and methods used have been described in the preceding paper (*11*) of this series.

* Part of this work was reported at the International Symposium on Enzyme Chemistry in Tokyo-Kyoto, in October, 1957.

RESULTS

Effect of Cyanide on Cytochrome a—Fig. 1 illustrates the effect of cyanide on cytochrome a in the reduced and oxidized forms. Addition of 0.01 *M* cyanide at pH 7.4 to ferricyanide-oxidized cytochrome a, caused the γ-peak at 424 mμ to shift to 428 mμ and to increase slightly in optical density. However, there was no change in absorption spectrum in the region of the α-peak. With dithionite-reduced cytochrome a, there is no shift in the position of the γ-peak on addition of cyanide, but its optical density decreases about 10 per cent. Also in the reduced state the α-peak is hardly effected by cyanide. The oxidized cytochrome a—cyanide complex can be reduced by dithionite or reduced cytochrome c and the resulting spectrum is the same as that of the reduced cytochrome a—cyanide complex. This result agrees with that of W a i n i o (*10*).

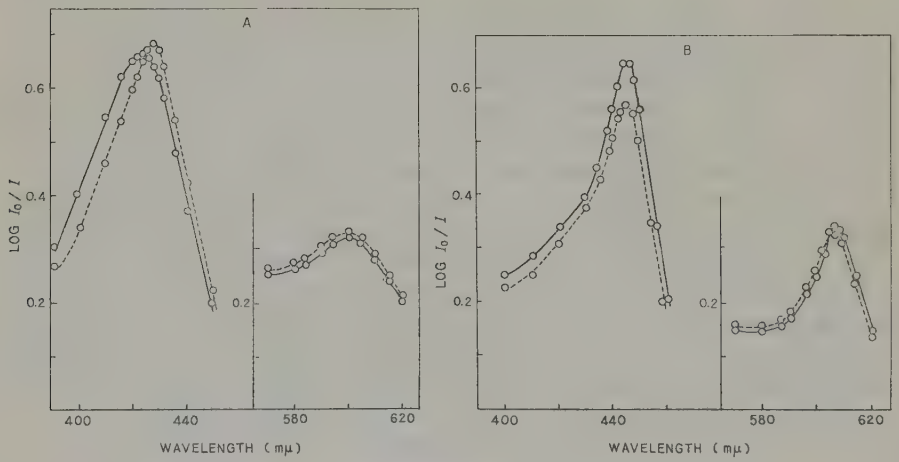

FIG. 1. Effect of cyanide on cytochrome a.

A. Effect of cyanide on oxidized cytochrome a.

B. Effect of cyanide on reduced cytochrome a.

Experimental conditions as described in the text. Oxidized and reduced cytochrome a were obtained by the addition of a small amount of potassium ferricyanide and sodium dithionite, respectively. The concentration of cytochrome a at which the spectrum of α-peak was obtained, was different from that for the spectrum of γ-peak. —○— cytochrome a, – –○– – cytochrome a in presence of cyanide.

Effect of Carbon Monoxide on Reduced Cytochrome a—Fig. 2 shows the absorption spectra of the dithionite-reduced form and the carbon monoxide complex of cytochrome a. In the presence of carbon monoxide, the γ-band shifts from 444 mμ to 430 mμ, but there is only a little change in the region of the α-band at 605 mμ. This is in agreement partially with the results of other investigators (*2–7*); the shoulder at about 444 mμ noted by these investigators, is not observed in our preparation.

Effect of Nitric Oxide on Reduced Cytochrome a—An effect of nitric oxide on reduced cytochrome a was first reported by Wainio (*10*). In his preparation the γ-peak at 443 mμ was devided into two peaks, on addition

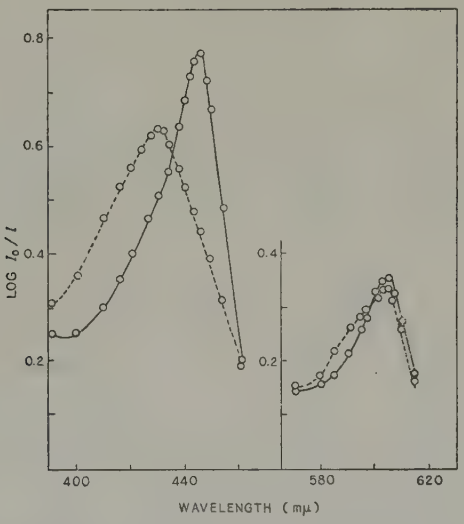

FIG. 2. Spectra of dithionite-reduced cytochrome a and its carbon monoxide complex.

—○— dithionite-reduced form of cytochrome a, – –○– – carbon monoxide-reduced cytochrome a complex.

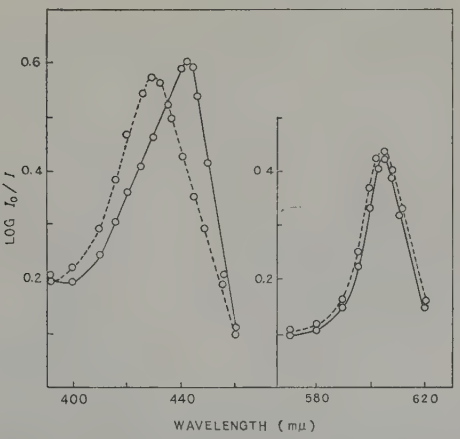

FIG. 3. Effect of nitric oxide on dithionite-reduced cytochrome a.

—○— dithionite-reduced cytochrome a, – –○– – nitric oxide-reduced cytochrome a complex.

of nitric oxide one at 439 mμ and the other at 430 mμ, while the α-peak at 605 mμ shifted to 603 mμ. In our preparation, the γ-peak shifts from 444 mμ

to 430 mμ with a slight decrease in optical density but twinning is not observed. In the region of the α-band, nitric oxide causes a slight increase optical density and the maximum shifts from 605 mμ to 603 mμ.

Effect of Oxygen on Reduced Cytochrome a—If the cytochrome a preparation is strongly autoxidizable, dithionite-reduced cytochrome a should be oxidized rapidly by oxygen, giving a spectrum of the oxidized form. As noted by Yakushiji and Okunuki (*8*, *9*), however, cytochrome a has little autoxidizability. This can also be seen in a cytochrome c—free particle preparation from heart muscle. Therefore we studied the effect of oxygen on reduced cytochrome a. When oxygen is bubbled through the dithionite-reduced cytochrome a preparation a new band appears (maxima at 426–28 mμ and 603 mμ) and the spectrum is clearly distinguishable from that of

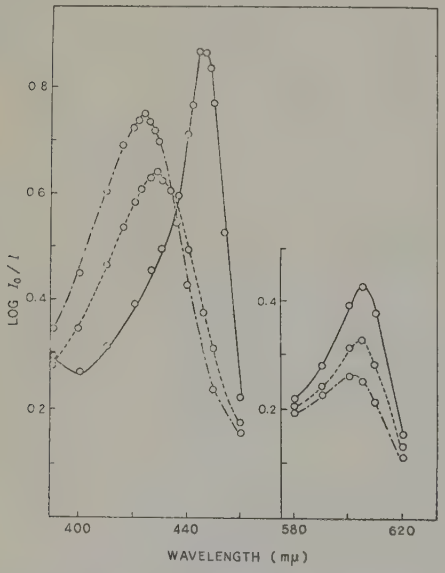

FIG. 4. Effect of oxygen on reduced cytochrome a.

—○— dithionite-reduced cytochrome a, - -○- - oxygen-reduced cytochrome a complex, ·—○—· ferricyanide-oxidized cytochrome a.

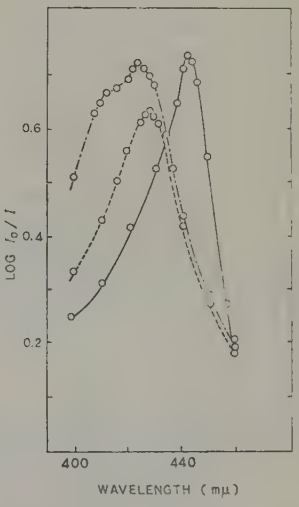

FIG. 5. Effect of oxygen on reduced cytochrome a in the presence of a small amount of cytochrome c.

—○— dithionite-reduced cytochrome a, - -○- - oxygen-reduced cytochrome a complex, ·—○—· oxygen-reduced cytochrome a in the presence of cytochrome c.

the oxidized form as illustrated in Fig. 4. This new band which appears by oxygenation is almost constant in our various preparations. This spectrum changes further to that of the oxidized form by the addition of a small amount of potassium ferricyanide. The spectrum of the oxygen-reduced cytochrome a complex is very similar to that of the carbon monoxide-cytochrome a complex in the region of the γ-peak, but differs in the region

of the α-peak. Thus this new spectrum suggests the oxygenation of cytochrome a in a way similar to hemoglobin or myoglobin.

As reported in the previous paper (*11*), our preparation has cytochrome oxidase activity and can oxidize reduced cytochrome c. On the other hand, there is no oxygen uptake when hydroquinone or ascorbic acid is added to our preparation in the absence of cytochrome c. Therefore, the action of oxygen on reduced cytochrome a was also examined in the presence of a small amount of cytochrome c. Fig. 5 shows the result obtained. In the presence of cytochrome c, reduced cytochrome a is rapidly converted to the oxidized form by oxygen. There is no change in the spectrum on further addition of ferricyanide. These findings will be discussed in detail in the next section.

It is generally agreed, especially in view of the competitive reaction of oxygen and carbon monoxide, that a specific characteristic of cytochrome oxidase is that it can react with oxygen. Therefore, the effect of mixture of oxygen and carbon monoxide on reduced cytochrome a was examined. We were not yet able to obtain a quantitative evidence that reduced cytochrome a can combine with oxygen and carbon monoxide in a competitive way, but the mixture (the ratio of the concentration of CO to O_2 being 1) caused a mixed spectra illustrated in Fig. 2 and 4 in the region of α-band. And, indeed, a competitive inhibition of cytochrome oxidase activity of cytochrome a preparation by carbon monoxide was observed manometrically.

DISCUSSION

The name cytochrome a_3 was first proposed by Keilin and Hartree (*1*) based on their spectroscopic observations of the effect of carbon monoxide and cyanide on a cytochrome oxidase preparation from heart muscle. Their hypothesis has been supported by more quantitative measurements of spectral changes of deoxycholate- and cholate-clarified heart muscle extracts by Straub (*2*), Ball, Strittmatter and Cooper (*3*), Dannenberg and Kiese (*4*), Stotz, Morrison and Marinetti (*5*) and Smith (*6*). In all instances, the spectrum of carbon monoxide-cytochrome oxidase complex has maxima at 430 mμ and 605 mμ, and obvious shoulders at about 444 mμ and 590 mμ. Based on these facts and the kinetic studies of Chance (*7*), they claimed that cytochrome a_3 is only one component of of the preparation and that oxygen and carbon monoxide react competitively with it in the respiratory system. However, Yakushiji and Okunuki (*8*, *9*) and Wainio (*10*) have argued against the existence of cytochrome a_3. They also studied the spectral properties of their purified preparations and were unable to find more than one component.

As reported in the preceding paper (*11*), our preparation of cytochrome a contains only one heme component and has weak cytochrome oxidase activity. The spectral properties of our preparation reported here also give no evidence for the existence of two α- and γ-peaks, which would be expected

if there were two components. As can be seen from Fig. 2, carbon monoxide modifies the spectrum of reduced cytochrome a as noted by many investigators mentioned above, but the shoulder around 444 mμ is not observed in our preparation. Other ligands such as cyanide, nitric oxide, or oxygen have a similar effect to carbon monoxide on cytochrome a. Thus, the observed reaction of cytochrome a with carbon monoxide or cyanide is almost the same as that of cytochrome a_3 noted by Keilin and Hartree. Based on these spectral properties of cytochrome a, it is doubtful whether cytochrome oxidase contains two heme components, namely a and a_3, and, indeed the existence of cytochrme a_3 has not yet been observed directly.

From the result of Yakushiji and Okunuki (*8*, *9*) it is known that cytochrome a is less autoxidizable if cytochrome c is absent. As shown in Fig. 4 oxygen modifies the spectrum of dithionite-reduced cytochrome a and a new spectrum appears which is clearly distinguishable from either the reduced or oxidized form of cytochrome a. On the other hand, when a small amount of cytochrome c is present in the system, reduced cytochrome a is rapidly converted to the oxidized form in the presence of oxygen as illustrated in Fig. 5. There is also a possibility that oxygen and carbon monoxide react competitively with cytochrome a. Thus the reduced form of cytochrome a is not oxidized by oxygen but forms an oxygenated complex. These findings suggest that cytochrome a functions as cytochrome oxidase in the presence of cytochrome c. The role of cytochrome c in this reaction is not yet clear, but perphaps it may play the role just as coenzyme against apoenzyme. In this connection, it is of special interest that cytochrome a, which is hardly affected by other reducing agents except dithionite and *p*-phenylene diamine, is easily reduced by reduced cytochrome c.

The formation of oxygenated complex of cytochrome in the course of cytochrome oxidation was postulated by Shibata and Tamiya (*12*). A similar action mechanism of cytochrome oxidase was suggested by Lemberg and Legge (*13*) by analogy with that of hemoglobin. If the oxygenated form of cytochrome a really exists, the cytochrome oxidase mechanism can be easily understood. The relation between cytochrome a and cytochrome oxidase will be studied in detail in the following paper.

SUMMARY

1. Potassium cyanide at a final concentration of 0.01 *M* causes a shift of the γ-band of oxidized cytochrome a from 424 mμ to 428 mμ, but no shift of that of the reduced form.

2. The γ-band of reduced cytochrome a shifts from 444 mμ to 430 mμ in the presence of carbon monoxide, while there is a small change in the region of the α-band.

3. Nitric oxide can combine with reduced cytochrome a and absorption maxima appear at 430 mμ and at 603 mμ.

4. The oxygen complex of reduced cytochrome a has maxima at

426–28 mμ and 603 mμ. The spectrum is clearly distinguishable from that of the oxidized form. In the presence of a small amount of cytochrome c, reduced cytochrome a shows a strong autoxidizability.

5. The function of cytochrome a as cytochrome oxidase is discussed on the basis of these findings.

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STUDIES ON PHOSPHOLIPID METABOLISM

VI. ON THE RATES OF INCORPORATION OF P^{32} INTO INDIVIDUAL PHOSPHOLIPIDS IN TISSUES

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(Received for publication, April 14, 1958)

In recent years studies on phospholipid metabolism have been carried out in our laboratory. As a work of this series, the rates of incorporation of P^{32} into individual phospholipids in various tissues were examined after its intraperitoneal administration.

In order to measure accurately the P^{32} labelling of individual phospholipids present in a small sample of tissues, it was tried to find out proper method of phospholipid fractionation.

Phosphatidylcholine has already been recognized to be separated from other (non-choline) phospholipids with magnesium oxide (*1*) or aluminum oxide (*2*). But in the method non-choline phospholipids cannot be separated furthermore.

Dawson (*3*) has recently found a method which could measure the labelling of P^{32} of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and diphosphoinositide in a small sample of tissue.

The procedure is: the lipids isolated from the tissue were hydrolysed with methanolic NaOH, and after the removal of alkali and fatty acids, the breakdown products of the phospholipids were resolved by two-dimensional filter paper chromatography.

Investigating and modifying his method, we found a method applicable to the study. With the method, the incorporation of P^{32} into various phospholipids in tissues, especially in liver, was measured at several intervals after intraperitoneal injection of labelled phosphate.

The results show that in rat liver higher specific radioactivity exists in diphosphoinositide fraction 30 minutes after injection, although phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine had also radioactivities, while after 12 hours the four phospholipids had the same specific activities.

METHODS

The labelled sample of tissue (300–500 mg.) was extracted with fifty volumes of

ethanol-ether (3 : 1). The extracts were evaporated to dryness under reduced pressure in nitrogen stream. The residue reextracted with 5–10 ml. of petroleum ether and 5 ml. of chloroform-methanol (3 : 1). The extracts combined were evaporated and the residue was then taken up in 0.8 ml. of carbon tetrachloride. To the solution was added 6.3 ml. of methanol and 0.9 ml. of water. The mixture was then treated with 2.0 ml. of 1 *M* methanolic NaOH and immediately incubated at 37° for 15 minutes. After cooling the solution in ice, the hydrolysate was diluted with 16 ml. of water (ice-cold) and the milky fluid quickly passed through a column of Amberlite IRC 50 resin (7 × 0.8 cm.). After washing thoroughly with 10 ml. of water the ice-cold hydrolysate was shaken successively with 25 ml. portions of carbon tetrachloride, ether and petroleum ether. The turbidity of the aqueous laver was removed by shaking with 2 volumes of *iso*-butylalcohol. The aqueous solution was filtered, neutralized to pH 7 with ammonia and evaporated to dryness under reduced pressure below 50°. The residue was taken up in a little water and applied to a filter paper chromatogram which was developed with phenol/NH_3 (18 hours) and *tert*-butanol/ trichloroacetic acid/water (20 hours). The spots were located by counting radioactivity and by spraying with the acid-molybdate reagent and ninhydrin reagent.

The radioactivities of the spots on the developed filter paper, located by acid-molybdate reagent, were counted by the Geiger tube.

Phosphorus contained in the spots was determined as follows: Two filter papers were developed at the same time. One of them was used to locate the spots with acid molybdate reagent, the other to estimate phosphorus in the spots. The latter, of which individual spot was fixed by comparing with the spot of the former, was cut into pieces according to the location of spots. Then, they were extracted with water in test tubes. After filtration, the clear fluid was evaporated to dryness, to which 1 ml. of HCl-$HClO_4$* solution was added. The mixture was oxidised and concentrated by heating for 15 minutes, then was cooled. One ml. of water, 1 ml. of 20 per cent sodium molybdate, 1 ml. of 1.5 *N* sulfuric acid and 4 ml. of isobutanol, were added into the test tube. After shaking this vigorously, it was left at room temperature until it divided into two layers. 2 ml. of isobutanol layer were transferred to the another tube, then 2 ml. of 0.2 per cent ascorbic acid solution and 1 ml. of ethanol were added immediately, and kept in a thermostat at 37° for 15 minutes. The optical density of the color developed was determined with a photometer.

Solvents for Chromatography: Phenol/NH_3; phenol saturated with 0.1 per cent (*w/v*) NH_3/ solution. *Tert*-butanol/trichloroacetic acid; *tert*-butanol, water 62/38 (*v/v*), trichloroacetic acid 10 per cent (*w/v*). After using this solvent, the paper was dried in air and trichloroacetic acid was removed by washing the paper in ether.

Ascending chromatography was used for the two solvents.

RESULTS

Mild Alkaline Hydrolysis of Phosphatidylcholine—When phosphatidylcholine, prepared from human brain and rat liver, was mildy hydrolysed with alkali and was treated as described above, there appeared only single spot on the paper chromatogram which was inseparable from glycerylphosphorylcholine (GPC). The water-soluble phosphorus derivative was completely hydrolysed in *N* HCl at 100° for 30 minutes to give glycerophosphoric acid (GP) which was chromatographically identified and choline which was precipitated as reineckate, and identified chromatographically.

* 60 per cent (*w/w*) $HClO_4$ 5 ml., 1 *N* HCl 10 ml. water 85 ml.

Under the condition above specified, phosphatidylcholine gave GPC in a yield of about 70 per cent of the theoretical.

Mild Alkaline Hydrolysis of Phosphatidylethanolamine—Mild alkaline hydrolysis of phosphatidylethanolamine isolated from human brain gave a water-soulble phosphorus derivative of ethanolamine. The spot of this product on the chromatogram with phenol/NH_3 was in accord with glycerylphosphoryl-ethanolamine (GPE).

On hydrolysis with *N* HCl at 100° for 30 minutes the phosphorus derivative was completely decomposed and GP and ethanolamine were chromatographically detected in the hydrolysate.

After mild alkaline hydrolysis for 15 minutes the yield of GPE was about 65 per cent of theoretical yield.

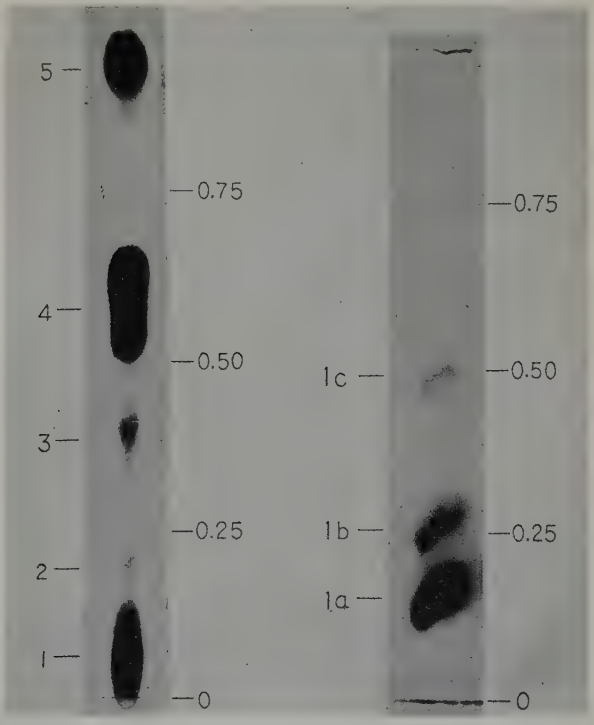

FIG. 1. Autoradiogram developed with phenol/NH_3 of the mild alkaline hydrolysate of P^{32} labelled lipides from rat tissues. 1. Glycerylphosphorylserine and hydrolysis product of diphosphoinositide (suggested by Dawson). 2, 3. Unidentified phosphorus containing substances. 4. GPE. 5. GPC.

FIG. 2. Autoradiogram developed with butanol/trichloroacetic acid of P^{32} containing products which existed in R_f 0.05–0.20 with phenol/NH_3. 1-a, Hydrolysis product of diphosphoinositide. 1-b, Glycerylphosphorylserine. 1-c, GP.

Mild Alkaline Hydrolysis of Lipids Extracted from Animal Tissues—The same procedure described above has been carried out on P^{32}-labeled lipids of rat

liver and other tissues.

The autoradiograms obtained are shown in Figs. 1 and 2. Fig. 1 shows chromatogram developed with phenol/NH_3 of the acid-soluble phosphorus-containing product derived from lipids. Fig. 2 shows a chromatogram of the hydrolysis products which located in the R_f range 0.05–0.20 with phenol/NH_3 and were reextracted with water from the paper with butanol/trichloroacetic acid.

The GPC and GPE are seen to be well separated from the other phosphorus-containing hydrolysis products in the phenol/NH_3 run. Two or three unidentified phosphorus-containing substances are found in Fig. 1.

In Fig. 2 three spots are found on the chromatogram. The slowest spot shows the strongest radioactivity and it seems to be in accord with the spot of hydrolysis product of diphosphoinositide according to Dawson's suggestion. The activity of the second spot, which seems to be Dawson's glycerylphosphorylserine, is very weaker than the slowest. The activity of the fastest one is very weak and it accords with glycerophosphate.

Inorganic phosphorus which contaminates lipids extracts could not be thoroughly removed by trichloroacetic acid treatment and shaking with 0.1 *N* HCl.

TABLE I

The Percentage of Phosphatidylcholine, Phosphatidylethanolamine and Other Phospholipids to Total Phospholipid in Rat Liver, Kidney and Small Intestine

Phospholipids	Percentage of individual phospholipids to total phospholipid					
	P determination			Radioactivity determination		
	Liver	Kidney	Small intestine	Liver	Kidney	Small intestine
Phosphatidylcholine	58	59	58	60	60	61
Phosphatidylethanolamine	22	25	25	21	24	27
Unknown phospholipid	8	5	3	6	4	2
Phosphatidylserine and diphosphoinositide	12	13	14	13	12	10

Application of the method of alkaline hydrolysis to determine the specific activities of the phospholipids in liver 30 minutes and 12 hours after the time of intraperitioneal injection of P^{32}.

Using this methods, the percentages of phosphatidylcholine, phosphatidylethanolamine and other phospholipids to total phospholipid in rat liver, kidney and small intestine 24 hours after intraperitoneal injection of P^{32} were estimated. The results are shown in Table I.

Lipids in rat liver were extracted with ethanol-ether (3 : 1) after intraperitoneal injection of P^{32} (10^7 c.p.m./100 g. of body weight) and were dried in nitrogen stream under reduced pressure. The residue was reextracted with petroleum ether and chloroform-methanol. The specific activities of the individual phospholipids were measured after they were hydrolysed with alkali and the hydrolysis products separated by paper chromatography. Table II shows the results obtained 30 minutes and 12 hours after the time of P^{32} injection.

TABLE II

Specific Activities of Phospholipids in Rat Liver after Intraperitoneal Injection of P^{32}

Phospholipids	Specific activities	
	30 minutes ($\times 10^3$)	12 hours ($\times 10^3$)
Phosphatidylcholine	3	110
Phosphatidylethanolamine	13	108
Phosphatidylserine (+ phosphatidic acid)	12.5	102
Diphosphoinositide	17	102

It is seen from Table I that 30 minutes after injection, diphosphoinositide fraction had been most actively labelled and phosphatidylcholine had showed the lowest specific activity, but after 12 hours every phospholipids had showed the equal specific activities.

DISCUSSION

Using solvent fractionation methods, Chargaff *et al.* (1940) (4) observed that lecithin was formed more rapidly in rat liver than did the cephalin fraction. In brain the cephalin and sphingomyelin had higher specific radioactivities than the lecithin fraction. A similar study by Hevesy and Hahn (1940) (*5*), and Hahn and Tyrén (1945) (*6*) showed that in rat and rabbit liver the turnover rate was considerable great in the cephalin fraction up to the twelfth hour. Thereafter the rate for lecithin and cephalin fraction became identical and remained so. These workers also showed that in brain the turnover rate for the cephalin fraction was greater than that for lecithin even after several days. On this basis they postulated the presence of at least two components in the cephalin fraction. The shingomyelin fraction, likewise, was observed to exhibit a rather rapid turnover rate. Entenman, Chaikoff and Frielander (*7*) found that specific activity time curves of non-choline-containing phosphatide and choline-containing phosphatide of liver are quite similar. Dawson (1954) (*8*)

observed that in guinea pig liver and brain the specific activity of methanol-insoluble fraction was higher than other lipid fractions in 3.4 hours and 23 hours after the intraperitoneal injection.

The incorporations of P^{32} into phospholipids in slices of brain and liver were also studied by Dawson, and of pancreas and brain cortex by Hokin and Hokin (*10*), using paper chromatographic separation method, and they recognized that the incorporations of P^{32} into various phospholipids were very dissimilar and the higher specific activities were measured in "phosphatidic acid" and diphosphoinositide. Dawson could not find radioactivity in other phospholipids, but Hokin observed the respectable incorporation in phosphatidylethanolamine in pancreas.

The present results show that in the intact rat liver the specific activities of the individual phospholipids were distinctly different in 30 minutes after intraperitoneal injection of P^{32}, but little different in 12 hours. The highest specific activity observed in diphosphoinositide fraction suggested by Dawson and the specific activities of phosphatidylethanolamine and phosphatidylserine (phosphatidic acid) were about 3/4 of that of diphosphoinositide. The specific activity of phosphatidylcholine was significantly low as 1/6. But, 12 hours after the injection of P^{32} the difference among the specific radioactivities of individual phospholipids could not be found.

Therefore, from these results, it was known that in the intact liver intraperitoneally injected P^{32} was incorporated faster into diphosphoinositide fraction than into the other phospholipids, especially phosphatidylcholine, but P^{32} incorporation into phosphatidylcholine gradually increased and the final incorporation into the all kinds of phospholipids reached to the similar level.

SUMMARY

1. The method reported by Dawson was investigated, and it was learned that it was able to determine the specific activities of phosphatidylcholine, phosphatidylethanolamine, diphosphoinositide and phosphatidylserine in small P^{32} labelled samples.

2. The method has been used to measure the incorporation of P^{32} into individual phospholipids in the intact rat liver after intraperitoneal injection of labelled phosphate.

3. After 30 minutes of the P^{32} injection, it was observed that the highest specific activity was in diphosphoinositide fraction, the specific activities of phosphatidylethanolamine and phosphatidylserine were about 3/4 of that of diphosphoinositide and the specific activity of phosphatidylcholine was significantly low as 1/6, but there was little difference among the specific activities of individual phospholipids 12 hours after the injection.

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SYNTHESIS OF γ-AMINO-α-HYDROXYBUTYRIC ACID

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Up to the present, in the synthesis of γ-amino-α-hydroxybutyric acid Fischer and Goddertz's method (*1*) (1910) has chiefly been used, the method in which starting substance, γ-phthalimido-α-bromobutyric acid is converted to γ-phthalimido-α-hydroxybutyric acid by replacing bromid at the α-position by hydroxygroup, and then by hydrolysis of this product γ-amino-α-hydroxybutyric acid is obtained. In this case the starting substance is prepared by coupling phthalimid kalium and acetic acid chloroethylester (*2*) but as acetic acid chloroester is a substance extremely difficult to obtain, the author started the synthesis with γ-aminobutyric acid, the substance which has become readily available of late. Succeeding in the prepartion of a quite fair amount of γ-amino-α-hydroxybutyric acid, the author presents here the results of the synthesis.

EXPERIMENTAL

1) N-Acetyl-γ-Aminobutyric Acid—To 10 g. of γ-aminobutyric acid placed in a 50 ml. round bottom flask, 10 ml. of acetic anhydride freshly prepared by distillation and a drop of conc. sulfuric acid are added, and under reflux condensor, it is heated for several hours on a hot water bath. After leaving this at the room temperature to cool, white crystals are formed. This precipitate is filtered and dissolved in minimized amount of absolute alcohol, and by adding several times the amount of ethylacetate to the solution and kept it in a cool room for 2 weeks, short crystals of plate-form can be obtained. The quantity of crystals obtained in the present experiment amounted to 11 g. whose m.p. was found to be 126–7°.

$C_6H_{11}O_3N$ Calcd. C 49.66, H 7.64, N 9.64 (per cent)
Found C 49.48, H 7.51, N 9.45 (per cent)

2) Bromination—Taking 10 g. of the substance obtained previously, possessing m.p. 126–7° and mixing it with 1.2 g. of red phosphorus, the mixture is placed in a 50 ml. round flask. Under cooling with ice, 12 ml. of bromide is poured gradually into this mixture with stirring all the while. When this process is completed, a long glass tube is connected to this flask as a gasket, and the flask is heated over a hot water bath for 2 or 3 hours until no more HBr-gas escapes from the glass tube. Later, brown, syrup-like reaction

product is formed but not almost crystalied.

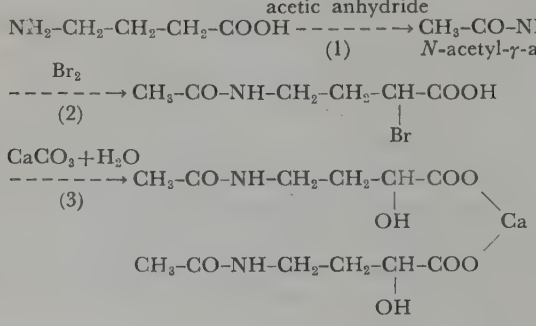
3) *Ca-salt*—The product obtained above is suspended in 500 ml. of hot water, and 24 g. of calcium carbonate is added to it in several portions. As CO_2 develop at each addition of $CaCO_3$, a suitable "bubble-eradicating" agent (in this instance 1 or 2 drops of octylalcohol) needs to de added beforehand. When the addition is finished, this is placed on a hot water bath in order to complete reaction; and by filtration excess amount of calcium carbonate is removed. Then the filtrate thus obtained is subjected to vacum distillation to dryness.

4) *Hydrolysis*—Ca-salt obtained above is put in 30 times the volume of 25 per cent hydrochloric acid, and heated on a boiling hot water bath for 12 hours. During the heating, a small amount of hydrochloric acid is added to it from time to time. When the reaction is completed, the reaction compound is subjected to vacum distillation to eliminate water completely; the resultant solid was extracted with 50 ml. of absolute alcohol by boiling for a few minutes. The residue is repeatedly extracted with 20 ml. of absolute alcohol for each time.

5) *γ-Amino-α-Hydroxybutyric Acid*—The above alcoholic extract was condensed to 20 ml. *in vacus* and then ethyl acetate is added to this until the solution becomes cloudy. When it is left in a cool room for a little while, it becomes transparent; and then it is made cloudy again by adding some more ethyl acetate. When this is left in a cool place, a brown oily precipitate appears at first; but when it is left in a refrigerated room for about 2 weeks, it solidifies itself and becomes white crystalline. This is separated and dried *in vacus*. The dried substance is again dissolved in small amount of absolute ethyl alcohol; and after adding charcoal to it and heating it for ten minutes, it is filtered. When the filtrate becomes cloudy by adding ethylacetate and left in a refrigerated room for two weeks, a white crystalline substance is obtained. By repeating this last process several times and by drying it in a desiccator containing concentrated sulfuric acid *in vacus*, deliquescent white crystals possessing m.p. 191–192.5° is obtained.

$C_4H_9O_3N$ Calcd. C 40.33, H 7.61, N 11.73 (per cent)
Found C 40.28, H 7.75, N 11.51 (per cent)

From these experimental procedures, the results as shown in the following table were obtained.



$$\xrightarrow[(4)]{HCl} NH_2\text{-}CH_2\text{-}CH_2\text{-}CH(OH)\text{-}COOH$$

γ-amino-α-hydroxy butyric acid

DISCUSSION

As described so far, the synthesis of γ-amino-α-hydroxybutyric acid has been attained by starting with γ-aminobutyric acid, and in this synthesis chemical reaction seems to have taken the following pathways.

At first acetylation is carried out in order to protect the amino-radical of γ-aminobutyric acid. This can be almost theoretically accomplished, as in the step of present experiment, by heating γ-aminobutyric acid with acetic anhydride of the same molar. The product of this reaction is quite soluble in alcohol and it no longer presents ninhydrin reaction. Barker (*3*) has also synthesized *N*-acetylaspartic acid by a similar method; and in the results of analysis, it is believed that *N*-acetyl-γ-aminobutyric acid has also been produced in the present experiment.

The next step is bromination. From the reaction point of view, *N*-acetyl-α-bromo-γ-aminobutyric acid seems to be produced through the replacement of bromine of *N*-acetyl-γ-aminobutyric acid.

The third step is heating it with calcium carbonate and water. It seems that in this reaction, α-position is hydroxylated and Ca-salt is produced by the combination of carboxyl radical with calcium; and neither in the step 2 nor in the step 3 it has been possible to crystallize the reaction products.

The step 4 seems to be the splitting of acetyl radical and calcium by hydrochloric acid. This is a similar method as the splitting of Ca from phthalimid radical, the method used by Fischer *et al.* (*1*) when they produced Ca-salt of γ-phthalimid-α-hydrolybutyric acid.

The substance obtained has the melting point of 191–2° and it is identical with the melting point of γ-amino-α-hydroxybutyric acid obtained by the synthesis starting with γ-phthalimido-α-bromobutyric acid. At the same time this can be verified as the same substance from the results of chemical analysis.

SUMMARY

The synthesis of γ-amino-α-hydroxybutyric acid has been accomplished in the following manner: first, in order to protect the amino radical of γ-aminobutyric acid the acetylation of the radical is undertaken; and then its α-position is temporarily replaced by bromine. And finally bromine is replaced by hydroxy radical.

Acknowledgement is due to Prof. Jinnai for his encouragement and painstaking proof-reading and to Dr. Kuroda of Okayama Nutrists' College for his invaluable advices, in preparing this manuscript.

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OCCURRENCE OF ARGINYLGLUTAMINE IN GREEN ALGA, CLADOPHORA SPECIES

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(Received for publication, June 10, 1958)

In the course of our studies on the distribution of guanidine compounds in the animal and the plant kingdoms, paper chromatographic analyses showed that an extractable unknown guanidine compound is present in certain green algae (*1*).

As to the naturally occurring arginine peptides, not a few papers have hitherto been appeared. However, these peptides have been obtained mainly as degradation products from polypeptides or proteins. Recently, Roche *et al.* (*2*), using chromatographic procedures, have reported that a muscle extract from a scorpion contained the Sakaguchi-positive compounds which appeared to be peptides of arginine with aspartic and glutamic acids.

In the present work, the author, basing upon the previous work (*1*), isolated an arginyl peptide in addition to arginine from the extract of a fresh-water alga, *Cladophora sp.*, and determined the structure of this peptide.

MATERIAL AND METHODS

Material—The green algae (*Cladophora sp.*) used in this study were harvested from a fresh-water pond in the botanical garden of the Faculty of Agriculture, Kyushu University, Fukuoka.

Methods for Detection of Amino Acid and Peptide—For this purpose, the following two procedures were employed.

Paper Chromatography: A portion of a solution to be tested was subjected to one dimensional descending chromatography on a strip of Toyo filter paper No. 5 B (1.5×45 cm.). The solvent systems used were *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2, by volume), *tert*-butanol-formic acid-water (70 : 15 : 15, by volume) and *n*-butanol-ethanol-water (4 : 1 : 1, by volume). After development, the strip was dried at room temperature and stained with the Sakaguchi reagent or ninhydrin.

Paper Electrophoresis: This procedure was carried out on a strip of Toyo filter paper No. 52 (2×25 cm.) with approximately 100 volts and about 3 mA/strip for 5 hours in presence of 1/15 *M* phosphate buffer of pH 6.8 by the horizontal strip method. The staining was performed by the same way as in the case of paper chromatography.

RESULTS

I. Preliminary Experiments on the Presence of Arginine and Its Peptide in the Material

It was found by the chromatographic and electrophoretic procedures that an aqueous extract from the material contained two Sakaguchi-positive substances as shown in Table I and Fig. 1. The chromatographic R_f value and electrophoretic behavior of the one of them agreed well with those of arginine, while the other was considered to be a peptide because of the formation of arginine, glutamic acid and ammonia after complete hydrolysis by acid. However, when the peptide was subjected to a partial hydrolysis, the formation of another peptide was observed which was composed of only arginine and glutamic acid.

II. Isolation and Identification of Arginine from the Algae

The algae freshly obtained were spread overnight to remove water at room temperature. About 1 kg. of the material was homogenized in a mixer for 5 minutes with 5 liters of water and then subjected to extraction for 30 minutes at about 80° with occasional stirring in a boiling water-bath, followed by filtration through cotton cloth. The residue was reextracted once more with 5 liters of hot water. From the combined extracts protein was precipitated with a sufficient amount of basic lead acetate and filtered off. The filtrate freed from lead was passed through a 5×40 cm. column of Amberlite IRC-50 (H-form) at a rate of 5 ml. per minute. After washing the column with 2 liters of water, the fraction containing arginine and its peptide was eluted with 3 liters of 2 *N* ammonia solution. The eluate was concentrated to about 50 ml. under reduced pressure at about 60°. When a sufficient amount of flavianic acid was added to the concentrated eluate after acidification with sulfuric acid, about 5 g. of mixed flavianates of these compounds crystallized out. The flavianates obtained here were separated from each other as follows.

When the mixed flavianates were poured into a small volume of hot water, the only hardly soluble flavianate of arginine (about 1 g.) remained almost undissolved. After separation, the flavianate was converted into hydrochloride in the usual manner. The hydrochloride recrystallized from water-ethanol mixture melted at 216–217° and gave a rotation $[\alpha]_D^{18}+21.3°$ (2 per cent in 5 *N* HCl). The yield was 120 mg. The analytical data showed the compound to be L-arginine monohydrochloride.

Analysis: L-Arginine monohydrochloride
$C_6H_{14}O_2N_4 \cdot HCl$ Calcd. N 26.59
Found N 26.41

III. Isolation and Characterization of the Peptide

The fraction containing the flavianate of the peptide separated from

arginine flavianate was allowed to crystallize in a refrigerator. The crystals obtained here were recrystallized several times from hot water, however, the crystallizations proceeded slowly in every case. About 3 g. of practically pure flavianate of the peptide were obtained. For further purification, the compound was converted at first into copper salt and then acetate as follows.

A solution of the peptide freed from flavianic acid was boiled together with copper carbonate and the filtrate was evaporated to dryness on a boiling water-bath. When the crude copper salt thus obtained was triturated with an adequate amount of water, the scarcely soluble light blue copper salt remained almost undissolved. The copper salt separated by filtration and that obtained further from the filtrate after similar treatment were combined together and dissolved in a dilute acetic acid. By passing hydrogen sulfide through the acetic acid solution the copper was removed. The filtrate was evaporated to a syrup under reduced pressure at abut 50° and the resulting syrup was crystallized from water-ethanol mixture. After recrystallization from aqueous ethanol, the crystals melted at 183° and showed a rotation $[\alpha]_D^{20}+16.9°$ (2 per cent in water). The yield was 350 mg. The analytical data of the product were well compatible with the composition assumed tentatively as arginylglutamine acetate.

Analysis: Arginylglutamine acetate $C_{11}H_{22}O_4N_6 \cdot CH_3COOH$
Calcd. C 43.08, H 7.23, N 23.19, Amino-N 3.87
Found C 42.86, H 7.36, N 23.02, Amino-N 3.84

IV. Complete Hydrolysis of the Peptide by Concentrated Acid

The peptide was hydrolyzed by heating under reflux for 20 hours with 6 *N* HCl and from the hydrolysate the excess hydrochloric acid was removed in the usual manner. As shown in Table I and Fig. 1, the hydrolysate of of the peptide contained two ninhydrin-positive substances, one of which was also Sakaguchi-positive. The ninhydrin-Sakaguchi positive substance had an R_f value and a pattern identical with those of arginine. The other only ninhydrin-positive substance was found to be glutamic acid from the chromatographic and electrophoretic behaviors. The presence of ammonia in the hydrolysate was detected with Nessler reagent.

Quantitative Determination of the Constituents—For quantitative determination of the constituents, a definite amount of the peptide was subjected to hydrolysis with 6 *N* HCl in a sealed tube at 110° for 20 hours. After removal of the excess hydrochloric acid, the hydrolysate was made up to a definite volume with water. An aliquot of the well mixed hydrolysate, on the one hand, passed through a column of Amberlite IR-400 (OH-form) and another aliquot, on the other hand, was treated through a column of Amberlite IR-120 (Na-form). The amount of arginine plus ammonia and ammonia only were estimated from the former effluent (free from glutamic acid) by using the ninhydrin colorimetric method of Troll and Cannan (*3*) and the Conway microdiffusion method modified by Fruton *et al.* (*4*), respectively. The difference between the quantity measured as arginine by

TABLE I

R_f Values of the Peptide and Thier Hydrolysis Products

Sample	R_f Value			
	n-Butanol-acetic acid pyridine-water*		*tert*-Butanol-formic acid water	
	Ninhydrin reaction	Sakaguchi reaction	Ninhydrin reaction	Sakaguchi reaction
Aqueous extract of the algae	—**	0.15 0.21	—**	0.23 0.27
Arginylglutamine	0.15	0.15	0.23	0.23
Complete acid hydrolysate of arginylglutamine	0.20	0.20	0.28 0.46	0.28
Enzymatic hydrolysate of arginylglutamine	(0.14)*** 0.17 0.21	0.21	(0.18)*** 0.32 0.27	0.27
Partial acid hydrolysate of arginylglutamine	0.20	0.20	(0.27)*** 0.36 (0.46)***	(0.27)*** 0.36
L-Arginine	0.20	0.20	0.27	0.27
L-Glutamic acid	0.21		0.46	
L-Glutamine	0.17		0.32	
L-Ornithine	0.14		0.18	
L-Arginyl-L-glutamic acid	0.19	0.19	0.36	0.36

* Agrinine, glutamic acid and arginylglutamic acid were not well separable by this solvent.

** Ninhydrin reaction was not carried out because of the difficulty of the separation of amino acid and peptide by one dimentional chromatography.

*** R_f value of barely detectable spot.

the former method and the apparent quantity of arginine equivalent to that of ammonia estimated by the latter method shows the real value of arginine. The ninhydrin colorimetric method was also employed for the determination of glutamic acid in the latter effluent (free from arginine and ammonia). As shown in Table II, arginine, glutamic acid and ammonia were present in equimolar quantities in the peptide.

Isolation and Identification of Arginine and Glutamic Acid—330 mg. of the peptide acetate was hydrolyzed by heating under reflux for 20 hours with 15 ml. of 5 *N* HCl. The hydrolysate freed from the excess hydrochloric acid was passed through a 1.2×12 cm. column of Amberlite IR-120 in the hydrogen form to adsorb all of the amino acids produced. The column was washed with 100 ml. of water, then eluted with 150 ml. of 2 *N* ammonia solution. The eluate was evaporated to dryness under reduced pressure. The residue was dissolved in 20 ml. of water and the resulting solution was passed again through another column of Amberlite IR-120 in the ammonium form to catch arginine only. After washing the column with 100 ml. of

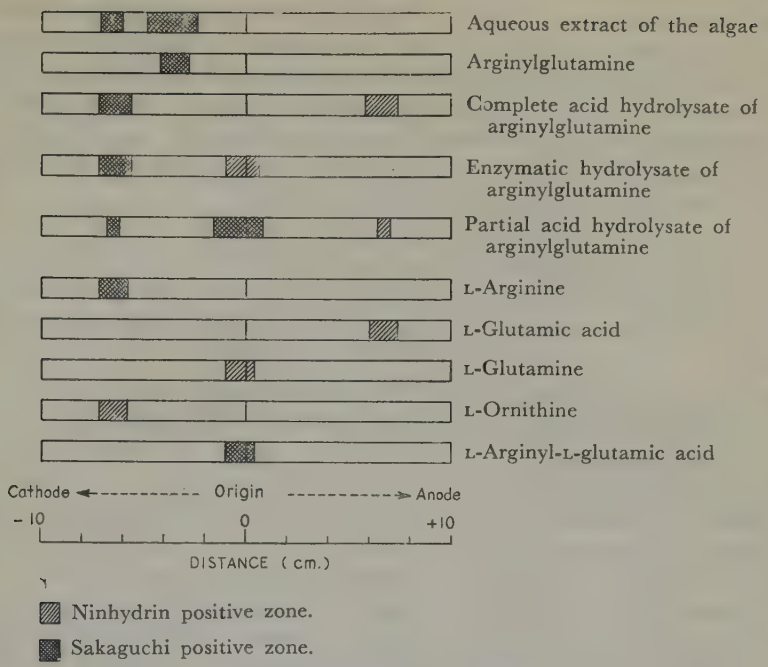

FIG. 1. Paper electrophoretic patterns of the peptide and their hydrolysis products. Staining reagent: Ninhydrin and Sakaguchi reagent.

TABLE II

Stoichiometry of Constituents in the Peptide

Peptide	Constituent		
	Arginine	Glutamic acid	Ammonia
0.189 μM	0.181 μM	0.182 μM	0.176 μM

water, the effluent and the washings were combined together and the mixed solution was concentrated to a small volume under reduced pressure. Upon addition of ethanol to it, glutamic acid crystallized. It was recrystallized from aqueous ethanol. The yield was 105 mg., m.p. 201°, $[\alpha]_D^{18}$ +30.5° (2 per cent in 5 *N* HCl). The analytical data showed the compound to be L-glutamic acid.

Analysis: L-Glutamic acid
$C_5H_9O_4N$ Calcd. N 9.25
Found N 9.42

On the other hand, arginine adsorbed on the column was eluted with 150 ml. of 2 *N* ammonia solution and the eluate was evaporated to

dryness under reduced pressure. The residue was dissolved in 10 ml. of water and the resulting solution, after neutralization with 0.1 *N* HCl, was evaporated under reduced pressure until a syrup obtained. When absolute ethanol was added to the syrup, arginine hydrochloride was obtained. It was recrystallized from water-ethanol mixture. The yield was 125 mg., m.p. 216-217°, $[\alpha]_D^{18}$+21.5° (2 per cent in 5 *N* HCl). The analytical data showed the product to be L-arginine monohydrochloride.

Analysis: L-Arginine monohydrochloride

$C_6H_{14}O_2N_4 \cdot HCl$ Calcd. N 26.59

Found N 26.47

V. Enzymatic Hydrolysis of the Peptide

As the elementary analyses of the peptide suggested the presence of glutamine as a constituent, its isolation was undertaken from an enzymatic hydrolysate of the peptide.

A dipeptidase used for this purpose was extracted from baker's yeast by the method of Grassmann and Klenk (*5*) and precipitated with acetone. After washing with acetone, the precipitate was dried *in vacuo*. The powdered precipitate was used as the enzyme preparation.

A buffered mixture (pH 7.8) consisting of the peptide and the enzyme was incubated at 37° for 24 hours. After incubation, protein was precipitated from the mixture with a sufficient amount of basic lead acetate and filtered off. A solution of the enzymatic hydrolysate freed from lead with sulfuric acid was then chromatographed, and the results are given in Table I and Fig. 1.

The chromatographic and electrophoretic results showed that three ninhydrin-positive substances were present, one of which was also Sakaguchi-positive. The ninhydrin-Sakaguchi positive substance gave an R_f value and a pattern identical with those of arginine. Of the two other only ninhydrin-positive substances the fairly detectable one showed the same chromatographic and electrophoretic behaviors as those of glutamine, while the other which was barely detectable had an R_f value identical with that of ornithine but its electrophoretic pattern could not be distinguished from that of arginine owing to the same basicity. These results indicate that after hydrolysis of the peptide to its constituents, a part of the produced arginine was further converted to ornithine and urea during incubation by the action of contaminated arginase.

Isolation and Identification of Glutamine—A mixture containing 300 mg. of the peptide acetate, phosphate buffer (pH 7.8) and 30 mg. of the enzyme powder was incubated at 37° for 24 hours. After incubation, glutamine was isolated from the deproteinized hydrolysate according to the method used for the isolation of glutamic acid from the complete acid hydrolysate of the peptide. The effluent and the washings from a column of Amberlite IR-120 (NH_4-form) was concentrated to a small volume under reduced pressure. When ethanol was added to it, glutamine crystallized. It was

recrystallized from aqueous ethanol. The yield was 85 mg., m.p. 185°, $[\alpha]_D^{18}$+6.8° (3 per cent in water). The analytical data showed the compound to be L-glutamine.

Analysis: L-Glutamine

$C_5H_{10}O_3N_2$ Calcd. C 41.09, H 6.90, N 19.17
Found C 40.89, H 6.88, N 18.86

VI. Sequence of the Constituents in the Peptide

In order to establish the sequence of constituents in the peptide, dinitrofluorobenzene method was used. The peptide was converted to its dinitrophenyl (DNP) derivative according to the method of Sanger (*6*).

TABLE III

R_f Values of DNP-Derivative of the Peptiae and Its Hydrolysis Products

Sample	R_f value (*n*-butanol-ethanol-water)		
	DNP-derivative	Ninhydrin reaction	Sakaguchi reaction
DNP-arginylglutamine	0.20		0.20
Hydrolysate of DNP-arginylglutamine	0.44	0.03	(0.01)* 0.44
α-DNP-arginine	0.43		0.43
L-Arginine		0.01	0.01
L-Glutamic acid		0.03	

* R_f value of scarcely detectable spot.

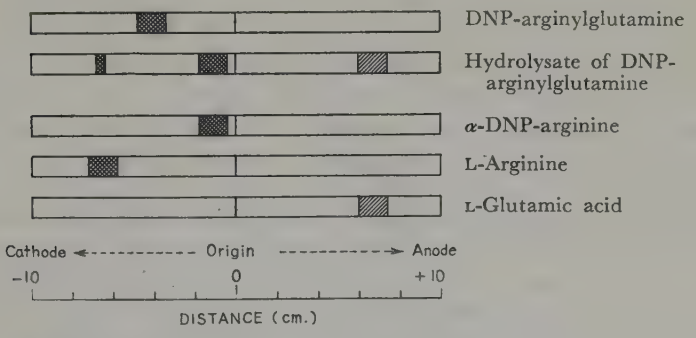

FIG. 2. Paper electrophoretic patterns of the DNP-peptide and its hydrolysis products. Staining reagent: Ninhydrin and Sakaguchi reagent.

The DNP-peptide was then hydrolyzed by heating under reflux for 8 hours with 6 *N* HCl. After removal of the excess hydrochloric acid, the hydrolysate

was evaporated to dryness under reduced pressure. The residue was dissolved in aqueous ethanol and the resulting solution was submitted to the chromatography and the electrophoresis. In these cases, yellow color of the DNP-derivative served for its detection. The results are given in Table III and Fig. 2.

The yellow constituent migrated to a position corresponding to DNP-arginine. A strongly colored spot and a slightly colored one with ninhydrin appeared in positions corresponding to glutamic acid and arginine, respectively. On the other hand, a spot stained deeply by the Sakaguchi reagent was superposed on the yellow spot occupied by DNP-arginine and another scarcely detectable one stained by the same reagent was seen in the position corresponding to the hardly detectable arginine spot with ninhydrin. These results indicated that although the smaller part of arginine was present in the free state, the greater part of it had been converted to the DNP-derivative. Consequently, it is concluded that arginine is the N-terminal amino acid of the peptide.

From this series of experiments, it may be concluded that the naturally occurring peptide in the green algae is L-arginyl-L-glutamine.

VII. Partial Hydrolysis of the Peptide by Dilute Acid

The peptide was hydrolyzed partially by heating under reflux for one and half hour with 1 *N* HCl. The hydrolysate was treated to remove the excess hydrochloric acid in the usual manner. A portion of the hydrolysate was then chromatographed. The results are shown in Table I and Fig. 1. There appeared three spots reacting with ninhydrin, two of which reacted also with the Sakaguchi reagent. The two spots in positions corresponding to arginine and glutamic acid were barely detectable, while the other one colored strongly with ninhydrin and Sakaguchi reagent. The latter was found to be a peptide yielding arginine and glutamic acid by further hydrolysis. The chromatographic and the electrophoretic behaviors of this compound agreed well with those of the synthetic arginylglutamic acid. On the other hand, ammonia was found to be liberated in equimolar ratio from the mother peptide by the hydrolysis.

Isolation and Identification of Arginylglutamic Acid—When a sufficient amount of flavianic acid was added to the hydrolysate from 500 mg. of arginylglutamine acetate, about 2 g. of the flavianate was obtained. After recrystallization from hot water, the flavianate was converted into free base in the usual manner. The resulting solution of free base was concentrated under reduced pressure until a syrup was obtained which crystallized by treatment with ethanol. The crystals were recrystallized from aqueous ethanol. The yield was 230 mg., m.p. 205°, $[\alpha]_D^{17}+20.5°$ (2 per cent in water). The analytical data agreed well with those of synthetic L-arginyl-L-glutamic acid monohydrate*.

* This compound was synthesized by the author *et al.* (*7*). L-Arginyl-L-glutamic acid monohydrate melted at 201–202° and showed a rotation $[\alpha]_D^{21}+20.9°$ (2 per cent in water).

Analysis: L-Arginyl-L-glutamic acid monohydrate
$C_{11}H_{21}O_5N_5 \cdot H_2O$ Calcd. C 41.11, H 7.22, N 21.80
Found C 41.16, H 7.10, N 21.65

SUMMARY

Paper chromatographic analyses of extracts from a fresh-water alga, *Cladophora sp.*, showed the presence of two Sakaguchi-positive substances, which was isolated by means of ion exchange resins. These substances were identified as L-arginine and L-arginyl-L-glutamine, a new compound. However, there was no evidence that L-arginyl-L-glutamic acid is present as a preformed peptide in the material.

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The Journal of Biochemistry, Vol. 46, No. 1, 1959

STUDIES ON THE METHYLATION OF PYRIDINE-COMPOUND IN ANIMAL-ORGANISMS[1-3]

II. PAPER CHROMATOGRAPHICAL DETECTION OF *N*-METHYL-PYRIDINE IN URINE OF RABBIT-ORGANISMS DOSED WITH PYRIDINE

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(Received for publication July 28, 1958)

It is known that the nitrogen of pyridine-ring is combined with methyl-group in animal body when pyridine is administered to them, although this combining power in rabbit-organisms is very weak (*1*, *2*). The author has made a study about the methylation-pattern for administrated pyridine in this case.

For this purpose, as His's conventional method is not available, it is most important to find the micro-analytical method of *N*-methyl-pyridine, so I have examined the qualitative and quantitative micro-detection of the *N*-methyl-pyridine by means of paper chromatography and ultra-violet absorption method.

EXPERIMENTS AND RESULTS

I. Preparation of Samples

Series I—Every day, the urine was collected from the rabbit administered 0.5 g. of pyridine by subcutaneous injection, and immediately evaporated to dryness in the electric oven at 55–60°. The each residue was extracted with 10 ml. of 85 per cent ethanol, filtrated, and the filtrate was used as samples.

Series II—Similarly, the urine was obtained from the rabbit administered 0.5 g. of pyridine *per os*, and was done by the same method as *Series I*.

The relationship between the samples and the materials is shown in Table I.

II. Paper Chromatography

For qualitative detections, 0.01 ml. of each sample-solution was chromatographed on Toyo filter paper No. 51, and run with the following solvents:

1) "Zur Kenntnis der Methylierung des Pyridins im Kaninchen Organismus" (*J. Biochem.*, **41**, 3 (1954)), is I of this series.

2) This work was supported in part by a resarch grant from the Scientific Research Funds of Ministry of Education.

3) Contributions from the Laboratory of Biological Institute Kobe University, Kobe, No. 56.

TABLE I

Time of the Day When Collecting Has Been Done and Quantities of Rabbit-Urine in Each Series

ate D 1957) (Sept.	Time of the day	Sample No.	Days elapsed after dosing	Quantities of rabbit urine	
				By injection *Series I*	By *per os Series II*
11th	6.30 p.m.	1	1	110 *ml.*)	— (*ml.*)
12th	9.00 a.m.	2	2	90	100
13th	2.30 p.m.	3	3	75	80
14th	10.00 a.m.	4	4	85	115
15th	6.00 p.m.	5	5	115	75

0.5 g. of pyridine was given to each rabbit at 4 p.m. September 10, 1957.

n-butanol : acetic acid : water = 4 : 1 : 2 or 4 : 1 : 5 *v/v* in mixture.

Then the dried chromatograms were sprayed with the next solution: 2 g. of *p*-aminobenzoic acid disolved in 5 ml. of 0.75 *N* HCl diluted to 100 ml. with 95 per cent ethanol. They are then exposed for one hour to CNBr vapors, the *N*-methylpyridine produced a pink-red spot, and it corresponds to that of *N*-methylpyridine which was separated by H i s 's method. This is a modification of CNBr-PABA treatment (*7*, *8*), and it is so sensitive that it can be detected up to μg. units of *N*-methylpyridine. The colored paper chromatograms of two series were shown in Fig. 1.

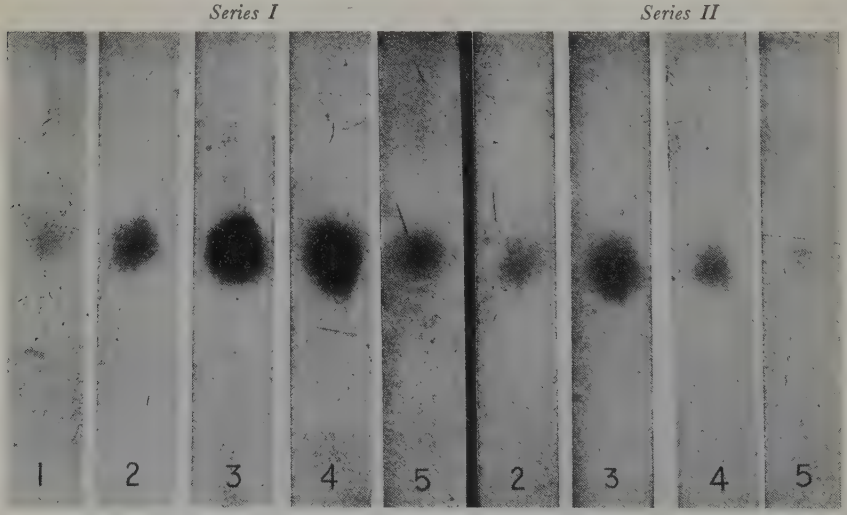

FIG. 1. Photograph showing the spots of *N*-methylpyridine in urine of rabbit administered with 0.5 g. of pyridine.

Series I; by injection, *Series II*; by *ber os.*

III. Quantitative Estimation

This paper chromatogram revealed that both the intensity of color of the spot and it's size varied with the quantity of the substance chromatographed. Thus, the estimation of *N*-methylpyridine in the samples was obtained by developing on chromatogram. In the practise, the large sheets of Toyo filter paper No. 51 are used, and the longedge of a sheet is marked with a series of small dots 3 cm. apart and 4 cm. from the edge to be inserted into the solvent. Each 0.01 ml. of the series of the samples was applied on the dots with capillary pippette.

For standard, take 0.1, 0.2, 0.4, 0.6, 0.8 and 1 ml. of 0.1 per cent *N*-methylpyridine solution in beaker respectively, add 10 ml. of 85 per cent ethanol, and were applied to the dots in the order of the concentrations.

And both chromatograms of the samples and the standard were developed, and colored with PABA-CNBr treatment.

Thus the quantity of *N*-methylpyridine in the rabbit urine was estimated, as shown in Table II.

Table II

Quantitative Estimation of N-Methylpyridine in Rabbit Urine by the Paper Chromatograms (in mg.)

Sample No.		1	2	3	4	5
Series	*I*	0.4	0.9	1.5	2.0	0.3
	II	—	0.3	1.5	1.5	0.2

IV. Spectrophotometric Estimation

The accurate quantitative procedure was performed by cutting out the section of the developed chromatogram which contains *N*-methylpyridine, and determining its amount in the elutriate by a Beckman spectrophotometer.

The curves such obtained show that the sample eluted by acetone was the most desirable for the spectrophotometric determination, and that the wave length of 329 mμ gives the greatest difference in ultra-violet absorption between the blank and the sample, as shown in Fig. 2.

Then the density of absorption was measured at the 329 mμ in wave length with 10 mm. absorption cells, by a Shimadzu Q. S. 20 spectrophotometer. The resultant absorbance was shown in Table III.

And the quantitative estimation of *N*-methylpyridine in rabbit urine was made with more accurate by absorbance-concentration.

For inspection of chromatogram, the relationship between the absorbance of ultra-violet absorption and the intensity of color of the spots was examined. One was paperchromatographed, and the other measured the density of ultra-violet absorption with same samples. The results were shown in Fig. 3.

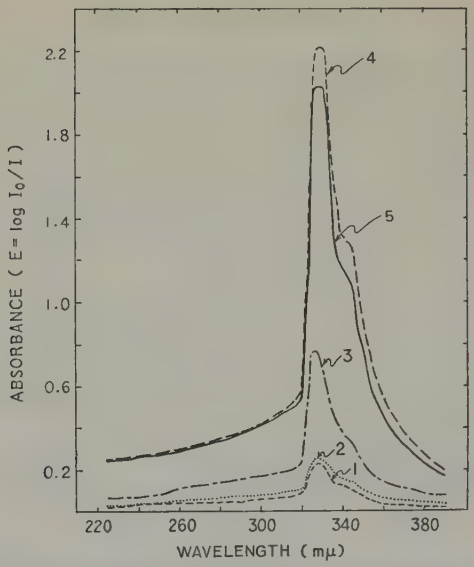

FIG. 2. Diagram illustrating the ultraviolet absorption curves of *N*-methylpyridine in urine of rabbit administered with 0.5 g. pyridine by injection.

TABLE III

The Density of Ultra-Violet Absorption of N-Methylpyridine in Rabbit Urine, and Its Estimated Quantity with Absorbance

Samples No.	*Series*	1	2	3	4	5
Absorbance (E=log I_0/I; wave length 329 mμ)	*I*	0.41	0.87	1.68	1.95	0.33
	II	—	0.26	1.89	1.46	0.24
The estimated quantity of *N*-methylpyridine (in *mg.*)	*I*	0.198	0.418	0.805	0.936	0.156
	II	—	0.125	1.907	0.701	0.116

DISCUSSION

For the preparation of the samples for detections of the metabolic products of pyridine, drying method is suitable, the procedure of which is simple, and the loss of the substance in the urine is minimum.

For paper chromatographical detection of *N*-methylpyridine, PABA (*p*-aminobenzoic acid)-CNBr treatment gave a satisfactory result.

This method is recommended for investigations on the metabolic pattern of pyridine-compounds, but the quantitative estimation can be more accurately obtained by the ultra-violet absorption method.

In the case of the quantitative estimation, the colored paper chromato-

grams show that the intensity of the resultant spots is proportional to the concentration of samples when the samples are applied to the paper to make the spots size constant (Fig. 3).

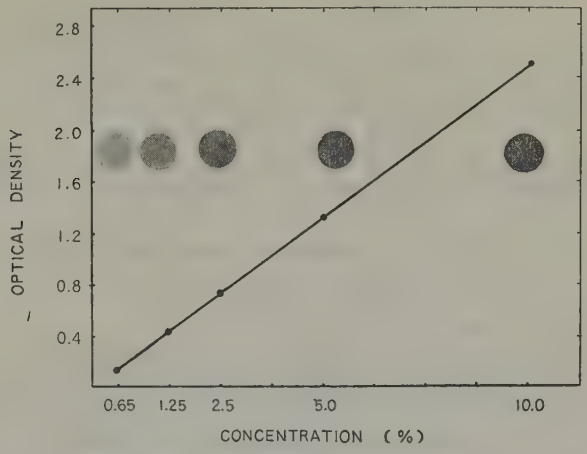

FIG. 3. Diagram showing the relationship between the density of ultraviolet absorption of *N*-methylpyridine and it's concentration, the intensity of colored spots.

SUMMARY

1. This report is a study on the qualitative and quantitative micro-detection of *N*-methylpyridine in urine, which was obtained from rabbit-organisms administrated with pyridine, the detection was done by paper chromatography and ultraviolet absorption with spectrophotometer.

2. The *N*-methylpyridine (Methylpyridyl-ammoniumhydroxide) can be qualitatively micro-detected by means of the paper chromatograms colored with PABA-CNBr treatment, and the spot is pink-red, and giving R_f values 0.4–0.5, run with solvent *n*-butanol : acetic acid : water = 4 : 1 : 2 or 4 : 1 : 5 (*v*/*v*) in mixture.

3. In the rabbit, *N*-methylpyridine has been excreted in urine during 5 days after pyridine was administrated, either in the case of injection, or *per os*.

4. The ultra-violet absorption spectra of acetone eluted *N*-methyl-pyridine in rabbit urine show the remarkable absorption maximum at 329 mμ in wave length (Fig. 2).

5. The quantitative estimation of *N*-methylpyridine in urine of rabbit-organisms dosed with 0.5 g. pyridine was due to the paper chromatograms and the absorbance of the ultra-violet absorption by a Beckman's spectro-photometer, were as follows:

The number of days after dosed with pyridine				1	2	3	4	5
The estimated quantity of *N*-methyl-pyridine (in mg.)	By visual comparison	*Serise*	*I*	0.4	0.9	1.5	2.0	0.3
			II	—	0.3	1.5	1.5	0.2
	By density of absorbance	*Series*	*I*	0.41	0.87	1.68	1.95	0.33
			II	—	0.26	1.89	1.86	0.24

The present writer is deeply indebted to Dr. M. Tomita who has given him kind encouragements and advices for the researches reported here.

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STUDIES ON CONJUGATION OF S^{35}-SULFATE WITH PHENOLIC COMPOUNDS

V. CONJUGATION OF SULFATE WITH PYRIDOXINE AND PYRIDOXAL IN RAT LIVER*

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(Received for publication, July 10, 1958)

It has been reported by Scudi *et al.* (*1*) that man and dog excrete the ingested pyridoxine in a conjugated form, but rat does not. Pyridoxine has been supposed to be conjugated with glucuronic acid or sulfuric acid through its phenolic hydroxyl group. Another metabolite has been isolated from the human urine, which has been identified with 4-pyridoxic acid (*2*).

In the present study the conjugation of S^{35}-sulfate with pyridoxine, pyridoxal and pyridoxamine in rat liver was examined and the conjugation of pyridoxine and pyridoxal with S^{35}-sulfate was confirmed.

EXPERIMENTAL

The method of incubation, paper chromatography and radioautogrphy is described in the preceding paper (*3*). Hydrolysis of the conjugated product on the paper was conducted by suspending the paper in hydrogen chloride fumes at room temperature for twenty minutes and then exposing it to currents of air.

CQC reaction was conducted as follows: The paper was sprayed with 0.1 per cent solution of 2,6-dichloroquinone-chlorimide in benzene and exposed to the vapours from boiling ammonia water (*4*). When the phenolic group of pyridoxine or pyridoxal is free, blue color appears immediately.

Diazo reaction was conducted as follows: The paper was sprayed with 10 per cent solution of sodium carbonate solution and then with diazotized sulfanilic acid solution.

The methods of identifying pyridoxine and pyridoxal component in the conjugates were those of Fujita (*5*) and Fujino (*6*). These methods were used after hydrolysing

* An account of the work described in this paper was presented at the 74th meetig of the Japanese Biochemical Society in Kanto District at the University of Tokyo on January 29, 1954. Part of it was also reported at the 26th general meeting of the Japanese Biochemical Society held in Sendai April, 1954.

the eluates of the conjugates in 1 *N* hydrochloric acid solution. The principle of these methods is as follows: After converting pyridoxine and pyridoxal to pyridoxic acid by specific treatments, the lactone of the acid is determined by fluorometry.

Attempts to synthsize the sulfate conjugates of pyridoxine and pyridoxal were made by the usual methods such as treating the compounds with chlorsulfonic acid in pyridine and chloroform (*7*) or moderately heatig them with pyrosulfate and alkali (*8*). The yield, however, by these methods proved poor when examined by color reactions on the paper chromatograms.

RESULTS

As shown in Fig. 1, when pyridoxine, pyridoxamine were incubated with S^{35}-sulfate in the presence of liver slices, pyridoxine and pyridoxal gave radioactive spots with respective R_f values of 0.27 and 0.19 on the paper chromatograms run in *n*-butanol—acetic acid—water (4 : 1 : 1). In another experiment run in water saturated butanol the R_f values were 0.12 and 0.10 respectively.

FIG. 1. Sulfate conjugates obtained in the presence of liver slices.

Liver slices 0.1 g.+ Ringer-phosphate-succinate (sulfate-free) 0.1 ml.+20 μg. of the substrate + S^{35}-sulfate, shaken in oxygen at 37.5° for 1 hour.

1, pyridoxine; 2, pyridoxal; 3, pyridoxamine; 4, without substrate.

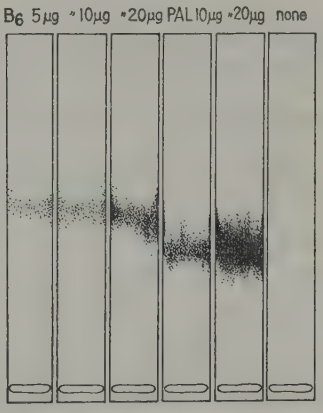

FIG. 2. The same as in Fig. 1. except that the amounts of the substrates added were varied.

B_6, pyridoxine; PAL, pyridoxal; none, without substrate.

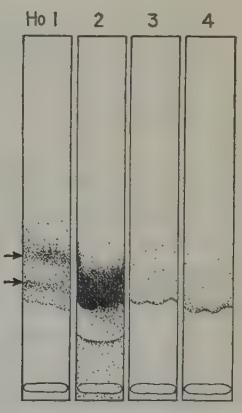

FIG. 3. Sulfate conjugates obtained in the presence of liver homogenate.

Homogenate (50 per cent) with KCl-Ringer-phosphate-succinate (sulfate-free) 0.1 ml.+K ATP 0.5 mg.+20 μg. of the substrate + S^{35}-sulfate, shaken in oxygen at 37.5° for 1 hour.

1, pyridoxine; 2, pyridoxal; 3, pyridoxamine; 4, without substrate.

The radioactivity of the conjugates varied in parallel to the amounts of the substrates added according to Fig. 2.

When the substrates were increased to 200 μg., these spots reacted with

CQC and diazo reagents to give colored compounds after the chromatograms were treated with acid. These colored spots coincided well with those of radioautograms and proved to be derivatives of either pyridoxine or pyridoxal by the tests of Fujita and Fujino.

In the experiments carried out with liver homogenate, similar results were obtained as shown in Fig. 3.

Pyridoxine gave, as shown in Fig. 3, two radioactive spots with R_f values of 0.27 and 0.19 run in *n*-butanol, acetic acid and water as a solvent which corresponded to sulfates of pyridoxine and pyridoxal respectively.

DISCUSSION

In the present study pyridoxine and pyridoxal have been shown to be conjugated with sulfate in the presence of rat liver slices as well as homogenate.

The position where sulfate conjugation took place was concluded to be in the phenolic group of the compounds because these conjugates showed positive color reactions characteristic to phenolic hydroxyl group only after the papers were treated in hydrogen chloride fumes.

When liver homogenate was used, pyridoxine gave pyridoxal sulfate in addition to pyridoxine sulfate. This indicates that pyridoxine is metabolized to pyridoxal in rat liver.

SUMMARY

A study has been made of the biological sulfate conjugation with pyridoxine, pyridoxal and pyridoxamine. Pyridoxine and pyridoxal have been shown to be readily conjugated with sulfate in the presence of rat liver slices as well as homogenate.

The position of the conjugation was concluded to be in the phenolic hydroxyl group.

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PHOPHORUS METABOLISM IN HUMAN ERYTHROCYTE

I. PAPER-CHROMATOGRAPHIC SEPARATION OF ACID-SOLUBLE PHOPHORUS COMPOUNDS INCORPORATING P^{32}*

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It has been generally accepted that the non-nucleated erythrocyte is not a mere container of hemoglobin, but a living cell in which rather active metabolic process is going on, coupled with the reduction of methemoglobin, the active transport of ions across the cell membrane and the other miscellaneous functions (*1–4*). Several investigations have been made to elucidate the metabolic pattern of the phosphorus compounds in mammalian erythrocyte. However, little information has yet been obtained on account of the technical difficulties in fractionation of the acid-soluble phosphate esters. Fleckenstein and Gerlach (*5*) and Prankerd and Altman (*6*) separated by the use of paper chromatography adenosine triphosphate (ATP), adenosine diphosphate (ADP), fructose-1,6-diphosphate (F-1,6-DP), 2,3-diphosphoglycerate (2,3-DPGA), and Bartlett *et al.* (*7*) analysed by means of ion exchange chromatography ATP, ADP, F-1,6-DP, 2,3-DPGA, hexose monophophate, and 3-phosphoglycerate (3-PGA).

The purpose of the present investigation was to obtain more detailed information about the constituents of the acid-soluble phosphorus compounds in human erythrocyte. The use of paper chromatographic procedure with the aid of P^{32} as described in the preceeding paper (*8*) permitted an effective separation of phosphate esters participating in the phosphorus metabolism in the cells.

METHODS

Five to twenty ml. of freshly drawn human venous blood was transferred into a vessel containing one tenth volume of 0.0033 *M* $Na_2HP^{32}O_4$ in 0.9 per cent NaCl solution with addition of 20 mg. heparin/100 ml. as anticoagulant. The amount of P^{32} was about 300 μc per 100 ml. blood.

The blood was incubated for 30 minutes at 37° with the gas phase of 5 per cent CO_2

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and 95 per cent air. At the end of the incubation period, twice the volume of ice-cold 0.9 per cent NaCl was added and the cells were immedately centrifuged down at 3,000 r.p.m. for 5 minutes at 0–5°. The plasma and the 'buffy coat' were removed, and the erythrocytes washed with ice-cold isotonic saline two times. The packed cells were extracted with ten volumes of ice-cold 10 per cent trichloracetic acid. The centrifuged precipitate was extracted again with two volumes of ice-cold 5 per cent trichloracetic acid. The combined supernatant fluids were carefully neutralized in an ice bath to pH 8.2 by addition of 10 *N* NaOH with phenolphthalein as an internal indicator.

The subsequent procedure was essentially the same as described previously (*8*) except slight modifications. One drop of 25 per cent barium acetate/ml. blood was added to the neutralized extract, and the barium salt of phosphate esters was precipitated by addition of four volumes of ethanol. The precipitate was suspended in a small amount of water and solubilized by treating with 0.1–0.5 ml. of Amberlite IR-120 (H form)/ml. blood. The supernatant was pipetted off and set aside, and the resin washed twice with minimum quantities of water. The supernatant and the washings were combined and again treated with Amberlite (0.1 ml./ml. blood). The clear supernatant fluid, centrifuged if necessary, was lyophilized and submitted to paper chromatography (*8*).

The chromatograms were run in following four solvents:

(A) Isopropanol-isoamyl alcohol-trichloracetic acid (5 per cent, *w/v*)-lactic acid 72 per cent, *w/w*), 15 : 5 : 10 : 0.5, according to Sekiguchi and Mano (*8*).

(B) Isobutyric acid-0.5 *N* ammonia, 20 : 12, according to Magasanik *et al.* (*9*).

(C) *n*-Propanol-concentrated ammonia-water, 20 : 10 : 3, according to Hanes and Isherwood (*10*).

(D) *tert*-Butanol-picric acid-water, 20 ml. : 1 g. : 15 ml., according to Hanes and Isherwood (*10*).

The esters were located by the use of ultraviolet light, radioautography, and spraying with coloring reagents. Sugars were detected by spraying with aniline hydrogen phthalate (*11*) or anisidine hydrochloride (*12*). For further examination, the individual spots were eluted with 0.1 *N* HCl into a test tube through capillary, and the eluates were tested for ribose by orcinol reaction (*13*), for fructose by resorcinol reaction (*14*), for glyceric acid by naphthoresorcinol reaction (*15*), and for purine by the reaction with diazobenzene sulfonic acid and sodium amalgam (*16*). Ultraviolet light absorption was determined on Hitachi EPU spectrophotometer after removal of the interfering impurities by extraction with freshly purified ether.

In order to demonstrate the acid-lability, the sample was hydrolyzed in 1 *N* HCl at 100° for 7 and 180 minutes, lyophilized, and submitted to paper chromatography.

For further identification of the radioactive compound the eluate of the individual spot was mixed with relatively large amount of an authentic sample, and the paper chromatogram was run. Agreement of the radioactive spot, in its shape and position, with the spot of known sample visualized by ultraviolet absorption or by coloring reagent is the proof of the identity.

RESULTS

Ten radioactive spots were obtained on the paper chromatogram run in the solvent A, as is shown in Fig. 1. These spots were numbered for convenience' sake in the order of increasing R_f values on the paper chromatogram. Some of the spots were separable by re-chromatography with other solvent systems. Thus, fifteen spots incorporating P^{32} were could be detected by

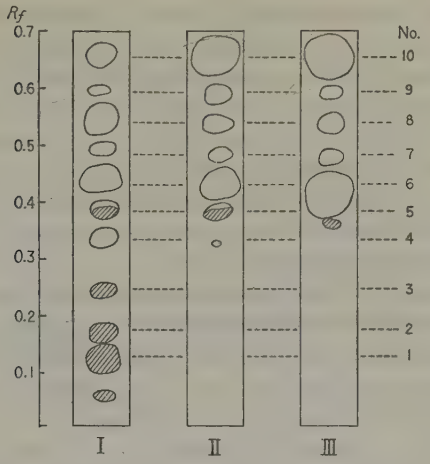

FIG. 1. Paper chromatograms of acid-soluble phosphorus compounds.

Erythrocytes were incubated with P^{32} at 37° for 30 minutes. The paper chromatograms were run in the sovent A, before (I) and after hydrolysis of the sample in 1 *N* HCl at 100° for 7 minutes (II) and 180 minutes (III). Radioactivity is indecated by empty areas, and ultraviolet absorption by shaded areas.

two-dimensional chromatography with solvent A and C.

Each of the spots shown in Fig. 1 was investigated as follows.

No. 1 (R_f=0.11). The eluate gave an ultraviolet spectrum identical with that of adenosine phosphate (maximum at 258 mμ), a red color with diazobenzenesulfonic acid and a positive orcinol reaction. Re-chromatography with the solvent B gave a main spot indistinguishable from ATP and two faint spots corresponding to ADP and inorganic phosphate. After hydrolysis in 1 *N* HCl for 7 minutes at 100°, no radio-activity was detected at the previous position on the chromatogram. From these findings this substance was identified as ATP.

No. 2 (R_f=0.17). The eluate possessed the same properties as those of the above spot except the R_f values. Further studies revealed that this adenine nucleotide corresponded in composition to adenosine tetraphosphate with two labile phosphates and two stable phosphates as will be reported elsewhere.

No. 3 (R_f=0.25). The spot was small and of the same properties as the spots No. 1 and No. 2, except that the R_f values in the solvent A and B were identical with those of ADP. Sometimes a very faint spot was observed a little below the main spot by radioautography as well as by the use of ultraviolet light, but no further investigation was made.

No. 4 (R_f=0.34). The eluate gave no ultraviolet absorption corresponding to purine or pyrimidine compound. After hydrolysis for 7 minutes it

faded from the paper chromatogram. The R_f value in the solvent A was very close to that of glucose-1-phosphate. However, when the eluate of the spot run in the solvent A was rechromatographed with the solvent C after mixing with an authentic sample of glucose-1-phosphate, the spot of the sample visualized by spraying with perchloric acid-molybdate reagent (*10*, *17*) was clearly distinguishable from the radioactive spots (Fig. 2 a). Re-chromatography in the solvent D of the eluate of this spot No. 4 gave three radioactive spots including that of inorganic phosphate (Fig. 2 g). The nature of this substance remains unclarified.

No. 5 (R_f=0.38–0.40). This spot absorbed ultraviolet light and gave a brown color with aniline hydrogen phthalate. Judging from its R_f value adenosine-5′-monophosphate (A-5′-P) and glucose-6-phosphate (G-6-P) were

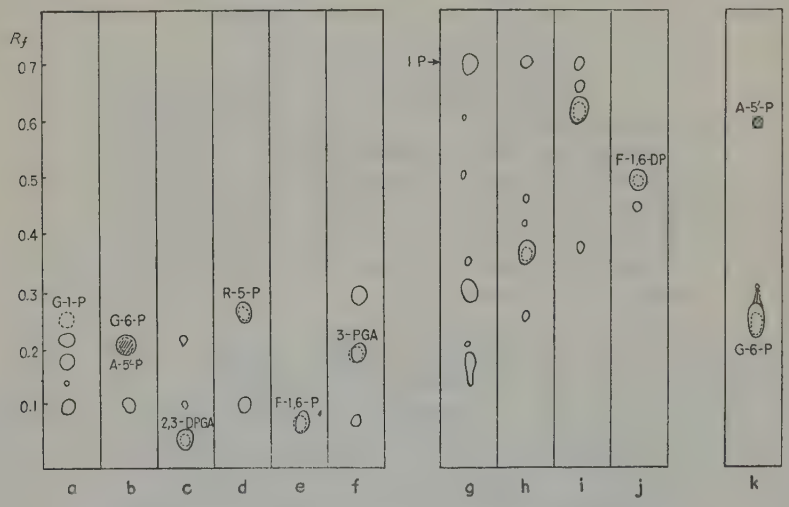

FIG. 2. Re-chromatograms of the spots in Fig. 1, I.

Radioacitvity is indicated by empty areas, and ultraviolet absorption by shaded areas. In some cases, the sample was mixed with a relatively large quantity of an authentic sample prior to the re-chromatography, and the spot was developed by spraying with perchloric acid-molybdate reagent. The colored spots are indicated by dotted lines. IP, the spot of inorganic phosphate. a-f, run in C; g-j, run in D; k, run in B.

suspected (*8*). When the eluate was re-chromatographed with the solvent B two spots were obtained, the one with R_f value of 0.60 and the other with R_f value of 0.25. The former possessed ultraviolet absorption and less radioactivity, while the latter no ultraviolet absorption and strong radio-activity. By re-chromatography after mixing with authentic samples adenosine-5′-phosphate and glucose-6-phosphate were identified ultimately. (Fig. 2 b and 2 k).

No. 6 (R_f=0.43). The spot was without ultraviolet absorption and characteristic in very active incorporation of radioactive phosphorus. By

spraying with perchloric acid-molybdate reagent (*11*, *17*) there appeared a large and intensive coloration correspoding to the radioactive spot. The eluate was resistent against acid hydrolysis for 180 minutes and gave positive reaction for glyceric acid with naphthoresorcinol. These findings and the R_f value in the solvent A suggest the compound is 2,3-diphophoglyceric acid. Final conclusion was reached by 'mixed' rechromatography with the solvents C and D (Fig. 2 c and 2 i).

No. 7 (R_f = 0.47–0.50). Several sugar phosphates locate about R_f=0.4–0.5 (*8*). The chromatogram of the eluate with added ribose-5-phosphate run in the solvent C gave a spot with R_f value of 0.26 by spraying with perchloric acid-molybdate reagent at the same position as the radioactive spot (Fig. 2 d). The eluate developed a brown color with orcinol in contrast with a green color of ribose-5-phosphate. Accordingly, there remains some uncertainty to conclude this spot is attributed to ribose-5-phosphate. There is a possibility, however, that a contamination with other sugar phosphates may result in a change of reaction color.

No. 8 (R_f=0.55). From the R_f values of the chromatograms (R_f=0.08 in the solvent C, and R_f=0.50 in D) and the red color developed by resorcinol reaction the spot was identified as fructose-1,6-diphosphate (Fig. 2 e and 2 j).

No. 9 (R_f=0.59). The spot was resistent to acid hydrolysis for 180 minutes. The R_f value in the solvent A was identical with that 3-phosphoglyceric acid (*8*). The rechromatogram of the eluate run in the solvent C showed two radioactive spots with R_f values 0.20 and 0.29. The former was identified with 3-phosphoglyceric acid by 'mixed' chromatography, and the latter was supposed most likely to be 2-phophoglyceric acid.

No. 10 (R_f=0.65). This spot is nothing but inorganic orthophosphate.

In addition to these ten spots, a radioactive substance with R_f value of 0.06 was detected on the chromatogram. No further investigation was, however, attempted to elucidate the nature of this compound.

DISCUSSION

In spite of the generally accepted view that non-nucleated erythrocyte has relatively poor matabolic activity, at least fifteen acid-soluble phosphorus compounds have been demonstrated to incorporate the added inorganic phophate-P^{32}. These compounds include ATP, ADP, AMP, G-6-P, F-1,6-D.P, 2,3-diphophoglyceric acid and 3-phosphoglyceric acid. In addition, the presence of a small amount of ribose-5-phosphate was suggested. Dische *et al.* (*18–20*) have demonstrated recently that when adenosine is added to hemolysate of erythrocyte, ribose-5-phosphate is produced and, when ribose-5-phosphate is added, it is converted into ribulose-5-phosphate and sedoheptulose-7-phosphate. From these data and the present observations, it is likely that hexose monophosphate shunt is operating in some extent physiologically in human erythrocyte, although the energy for the maintainance of the cellular functions seems to be supplied mainly through glycolytic process as indicated by active turnover of phosphorus in the members of Embden-

Meyerhof's system.

Gourley (*21*) and Bartlett and Marlow (*22*) have suggested the presence of glucose-1-phosphate in human erythrocytes, but this could not be confirmed in this laboratory.

A slight but distinct P^{32} incorporation was observed in adenosine-5'-monophosphate. This fact suggests that the nucleotide is synthesized in intact erythrocytes. Since the occurrence of adenosine deaminase, nucleoside phosphorylase (*23*, *24*), and nucleoside pyrophosphorylase (*25*) in erythrocytes has been demonstrated, the following pathway may be proposed:

$$\text{Adenosine} \longrightarrow \text{Inosine} + NH_3$$
$$\text{Inosine} + H_3PO_4 \rightleftarrows \text{Hypoxanthine} + \text{Ribose-1-phosphate}$$
$$\text{Ribose-1-phosphate} \rightleftarrows \text{Ribose-5-phosphate}$$
$$\text{Ribose-5-phosphate} + \text{ATP} \longrightarrow \text{Phosphoribosyl pyrophosphate}$$
$$\text{Adenine} + \text{Phosphoribosyl pyrophosphate} \rightleftarrows \text{Adenosine-5'-phosphate}$$

A new adenosine nucleotide with R_f of 0.17 resembles adenosine tetraphosphate described by Marrian (*26*) and Lieberman (*28*), but different from the latter in the number of acid-labile phosphate groups, as will be reported elsewhere. Active incorpotation of P^{32} suggests that this compound is playing an important role in the metabolism in erythrocytes.

SUMMARY

Acid-soluble phosphorus compounds of human erythrocytes were analysed with the aid of paper chromatography and P^{32}. After 30 minute incubation of erythrocytes with P^{32} at 37° at least 15 radioactive compounds were obtained, including ATP, ADP, AMP, G-6-P, F-1,6-DP, 2,3-diphosphoglyceric acid, and 3-phosphoglyceric acid as identified. The presence of ribose-5-phosphate was indicated. An adenine nucleotide with two stable and two labile phosphate groups was also found.

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ON THE N-TERMINAL PEPTIDE OF TAKA-AMYLASE A

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In the analytical studies on Taka-amylase A (*1*) it has been reported that as N-terminal residue one mole of alanine was demonstrated per mole of the enzyme protein, its molecular weight being assumed to be 52,000.

The determination of C-terminal amino acids of Taka-amylase A has been worked out by Ikenaka (*2*); he found each one mole of glycine, alanine and serine per mole of the the enzyme protein. It has been suggested, therefore, that Taka-amylase A has a branched chain structure.

The present paper deals with the studies on the amino acid sequence in the N-terminal peptide group of Taka-amylase A. There are several methods proposed for the stepweise degradation of peptides or proteins from their N-terminal group, (*2*, *3*) but none of them is repeatedly applicable to to natural proteins. Sanger's original dinitrophenyl (DNP) technique. that is partial hydrolysis of dinitrophenylated proteins followed by the fractionation of resulting dinitrophenyl peptides and characterization of each dinitrophenyl-peptides seems to be most suitable for practical use. Therefore, the present authors tried to obtain N-terminal DNP-peptides by a mild hydrolysis af DNP-Taka-amylase A by means of hydrochloric acid, but no N-terminal DNP-peptide could be obtained except DNP-alanine. It was found later that by heating DNA-Taka-amylase A with 80 per cent formic acid at 100° a number of DNP-alanyl-peptide were liberated,

One fifth g. of DNP-Taka-amylase A was digested with 9 ml. of 80 per cent formic acid at 100° for 10 hours. The lysate was concentrated *in vacuo* below 50°, added with dilute hydrochloric acid and extracted with ethyl acetate. The upper layer was shaken with 2 per cent sodium bicarbonate solution. The aqueous layer was acidified with hydrochloric acid and again extracted with ethyl acetate. DNP-peptide mixture obtained from the ethyl acetate extract was fractionated on silica gel column using various solvent systems. By these procedures three DNP-derivatives, P_1, P_2 and P_3, have been obtained in fairly pure state. P_1 was identified as DNP-alanine. Fractions P_2 and P_3 were hydrolysed separately by hydrochloric acid and amino acids formed were characterized by paperchromatography. C-Terminal amino acids were determined by hydrazinolysis as previously reported (*5*). The results are summarized as follows.

DNP-peptide	N-terminal	non-N-terminal	C-terminal
P_2	Ala	Gly	Gly
P_3	Ala	Gly, Asp	Asp

From these results it could be suggested that N-terminal peptide of Taka-amylase A has the following amino acid sequence.

(H) Ala-Gly-Asp-

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CHROMATOGAPHY OF CRYSTALLINE TAKA-AMYLASE A ON A WEAK ANION EXCHANGER

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(Received for publication, August 30, 1950)

The crystallization of Taka-amylase A (TAA) from "Takadiastase" (Sankyo) has been achieved by Akabori *et al.* (*1*).

Recently it was shown that most of the crystallized proteins were not absolutely pure, and that some chromatographic techniques enabled to fractionate crystallized proteins into more than one component, especially by using ion exchanger columns (*2–6*).

The present communication deals with the chromatographic study of crystallized TAA, using a weak anion exchanger to purity it further.

As the exchanger, Duolite A2 was employed, which contains primary, secondary and tertiary amine residues and is generally used for industrial decolorizing procedure. The eluent used consisted of sodium acetate aqueous

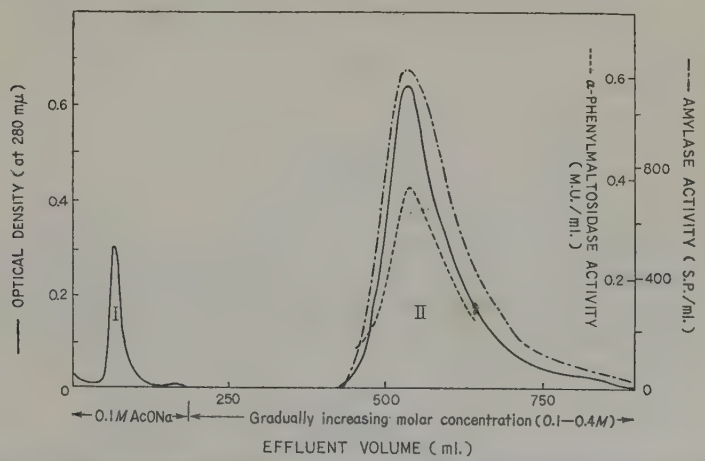

FIG. 1. Chromatography of caystalline Taka-amylase A. Culumn; Duolite A2 (60–240 mesh) 31 × 2 cm. Temperature; 12°. Flow rate; 35 ml./hour. Sample; 2 ml. of 2 per cent Taka-amylase A (recrystallized three times). Eluent; 0.1 *M* sodium acetate (200 ml.), gradually increasing molar concentration from 0.1 *M* sodium acetate (300 ml.) to 0.4 *M* sodium acetate (500 ml.). All eluents contain 0.01 *M* calcium acetate.

solution of gradually increasing molarity, starting at 0.1 *M* and approaching 0.4 *M*. In order to prevent TAA from denaturation, the eluent contained 0.01 *M* calcium acetate. A typical run is shown in Fig. 1. Absorbance at 280 mμ and enzymatic activities were measured with each fraction, at 30°. In measuring amylase activity, amylose (*7*) and α-phenyl-maltoside (*8*) were employed as the substrates. Maltase activity was also examined using maltose as the substrate (*9*, *10*).

As the figure indicates, one peak (I) was found after the column volume which had an ultra violet absorption at 280 mμ but not amylase activity.

Since a small maltase activity was found in this fraction, this fraction seems to contain maltase besides other proteins or peptides. Calculated from the optical density, its yield was 6 per cent. As to the main peak (II) which was eluted with 0.3 *M* of sodium acetate, it can be seen that this peak coincides with the peaks of both amylase and α-phenyl-maltosidase activities. The yield, calculated from the optical density and amylase activity, was about 75 per cent and 95 per cent respectively; this fact means that the specific activity increased about 25 per cent (saccharifying power per optical density at 280 mμ), or 10 per cent (saccharifying power per protein nitrogen).

TABLE I

Enzymatic Activities and Yield for Each Fraction

Peak	Maltase activity	Amylase activity (S.P./*mg.* N)	Yield	
			From optical density at 280 mμ	From amylase activity
Starting material	±	25000	—	—
I	±	0	*ca.* 66%	0
II	—	28000	*ca.* 75%	*ca.* 95%

The present result suggests that the weak anion exchanger, Doulite A2, may be employed for the preparation not only of crystralline TAA but also of other proteins. The adsorption capacity of Duolite A2 was about 25 mg. of TAA per milliliter of resin bed (in 1/10 *M* sodium acetate) or 65 mg. of TAA per gram of dry weight.

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NICOTINOTRANSFERASE FROM MYCOBACTERIUM AVIUM

As has been known in the literature, transferase catalyzes the exchange of the β-aspartyl group in asparagine and the γ-glutamyl group in glutamine with hydroxylamine or labelled ammonia in mammalian tissues (*1*), green plants (*2-4*) and bacteria (*5-8*), while this report will for first the time indicate the enzymatic transfer reaction of nicotinyl group in nicotinamide to hydroxylamine in *Mycobacterium avium*.

Mycobacterium avium (strain Takeo) was grown in glycerolbouillon medium for 4 days. The washed cells were poured into 10 volumes of acetone previously cooled to −30° by addition of dry-ice. After brief stirring, the cells were allowed to settle, the supernatant fluid was decanted and the filter cake of cells was desiccated in vacuo until the solvent was completely evaporated. Five grams of the acetone-dried cells were then finely ground and powdered with the aid of quartz sand and subsequently extracted with 300 ml. of 1 per cent sodium bicarbonate. After standing overnight in refrigerator, the cells were centrifuged off at 10,000 r.p.m. for 20 minutes at 0°. Four ml. of 1 per cent protamine sulfate solution were added to the crude extract and after stirring the precipitates formed were centrifuged off. The supernatant fluid was fractionted by the addition of solid ammonium sulfate, the fraction between 35 to 100 per cent saturation of ammonium sulfate was collected, and then dialyzed.

The enzyme assay was carried out by the measurement of the rate of hydroxamate formation according to the method of Lipmann and Tuttle (*9*).

When the activity of the enzyme towards amides was measured by the rate of hydroxamate formation, nicotinamide was found to be active, while glutamine and asparagine were inactive in various preparations as shown in Table I, Acetamide had slight activity in crude extracts. No hydroxamate was formed from a system containing nicotinic acid and hydroxylamine nor another system containing nicotinic acid, ATP and hydroxylamine in either persence or absence of Mg^{++} ion.

Table II indicates that each of Hg^{++} and Cu^{++} ion strongly inhibited the formation of hydroxamate, but Ni^{++}, Ca^{++}, Co^{++}, Ba^{++}, Mn^{++}, Mg^{++}, Zn^{++} and Cr^{+++} ions had no effect. This enzyme was not inhibited by some of metal chelating agents.

In summary, the results indicate that the extracts of *Mycobacterium avium* possess strong nicotinotransferase which catalyzes the formation of nicotinohydroxamate from nicotinamide and hydroxylamine, while aspartotransferase, glutamotransferase and synthetase catalyzing the hydroxamate formation

TABLE I

Hydroxamate Formation from Nicotinamide, Asparagine Glutamine and Acetamide

Enzyme \ Substrate	Nicotinamide 50 μM	L-Asparagine 50 μM	L-Glutamine 50 μM	Acetamide 100 μM
Crude Extract, 1260 μg.	4.14 μM	0.09 μM	0.00 μM	1.81 μM
Ammonium Sulfate (35%) 413 μg.	0.27	0.00	0.04	0.21
Ammonium Sulfate (100%) 223 μg.	1.07	0.01	0.00	0.12
Heated Enzyme*	0.00	0.00	0.00	0.00
Without Enzyme	0.00	0.00	0.00	0.03

* Boiled for 5 minutes at 100° (Ammonium Sulfate 100 per cent Fraction) Substrate as indicated; NH_2OH, 200 μM; K-phosphate buffer (pH 6.8), 200 μM; enzyme solution. Total volume, 4.0 ml.; 38°. 60 minutes.

TABLE II

Effect of Metal Ions on Transferase Activity

Metal		Hydroxamate Formation
None		1.50 *M*
Hg^{++}	1.25×10^{-4}	0.19
Cu^{++}	5×10^{-4}	0.05
Ni^{++}	5×10^{-4}	1.73
Ca^{++}	5×10^{-4}	1.69
Co^{++}	5×10^{-4}	1.68
Ba^{++}	5×10^{-4}	1.64
Mn^{++}	5×10^{-4}	1.61
Mg^{++}	5×10^{-4}	1.68
Zn^{++}	5×10^{-4}	1.66
Cr^{+++}	2.5×10^{-4} *M*	1.54

Nicotinamide, 50 μM; NH_2OH, 200 μM; K-phosphate buffer (pH 6.8), 200 μM; enzyme (ammonium sulfate 100 per cent fraction) 233 μg. Total volume, 4.0 ml.; 38° 60 minutes.

from aspartic, glutamic or nicotinic acid in the presence of ATP and hydroxylamine were not found. Since the nicotinamidasd was inhibited by Hg^{++}, Cu^{++}, Zn^{++}, Cr^{++}, and Fe^{+++}, it is quite apparent that this new nicotinotransferase in different from nicotinamidase purified 250-280 fold by Kimura (*10*) in respect of their behavior towards metal ions. The facts that *Mycobacteria* possess the strong nicotinamidase and nicotinotransferase will suggest the important role of nicotinamide in the metabolism of these bacteria. This concept seems to be supported by the findings that (I) isonicotinic acid hydrazide has been of therapeutic use for tuberculosis, (II) the administration of nicotinamide to the mice infected artificially with tuberculosis affected the survival of 90 per cent as compared with that of

5.3 per cent for untreated control (*11*), and (III) di-, α- and δ- nicotinyl-ornithine, nicotinuric acid and β-nicotinyl-D-glucuronide were found to be excreted from carboxy-labelled nicotinamide by chick (*12*).

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ISOLATION OF AN UNKNOWN AMPHOLYTE FROM THE URINE OF HYPOTHYROID PATIENTS

Several unusual amino acids found in the urine of hyper-and hypothyroid patients were already identified by Sonoda (*1*, *2*). But one ampholyte, which resists acid hydrolysis and has the R_f values on paper chromatogram: 0.60 (butanol-acetic-water) and 0.45 (phenol-water), is still unknown. This ampholyte appeared not in all the urine of these patients, but it appears most frequently in the hypothyroid patients. Recently this unknown compound was isolated in a crystalline form.

Fifteen liters of the urine of several hypothyroid patients were collected under toluene and desalted by the method of Carsten (*3*), and hydrolyzed in 5 *N* HCl for 24 hours at 100°. The ampholyte fraction thus obtained was at first fractionated on cation exchanger (Diaion SK-1) by 2 *N* HCl and then on anion exchanger (Dowex-1) by 0.1 *N* HCl. Each fraction, in both cases, was analyzed by one dimensional paper chromatography.

The conbined fraction containing the objective ampholyte was finally fractionated on a cellulose column by *n*-butanol-acetic-water (25 : 6 : 25 *v/v*) and the elute was collected in 5 ml. with the aid of a fraction collector.

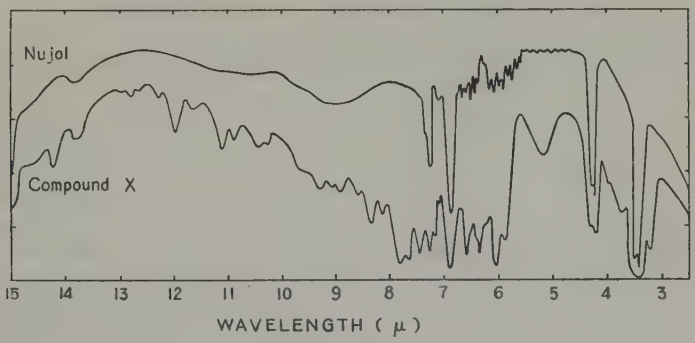

FIG. 1.

After chromatographic analysis on paper, the fractions containing the objective ampholyte were combined and evaporated to dryness in vacuum. The residue was taken up in a small amount of hot water. After treatment with charcoal, Ag_2CO_3 and H_2S, the free ampholyte was recrystallized several times from 50 per cent ethanol. 3 mg. of the crystals of rhombic plate were obtained. It melted at 232° leaving a slight brown colored liquid. Ninhydrin reaction on paper gave blue color. C per cent and H per cent were 43.4 and 6.7 respectively. The amount of crystals was not enough to analyze N per cent. The infra-red spectrum is shown in Fig. 1. The chemical

structure has not been elucidated. An attempt is being made to isolate more crystals.

Appreciation is expressed to Dr. T. Matsukawa (Takeda Research Laboratory) and Dr. K. Takeda (Shionogi Research Laboratory) for the elemental and infra-red analysis.

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HIGH CREATINE PHOSPHOKINASE ACTIVITY OF SERA OF PROGRESSIVE MUSCULAR DYSTROPHY

Creatine phosphokinase is one of the most abundant soluble enzymes in skeletal muscle (*1*). Considering the fact that the aldolase activity in serum increases remarkably in patients with progressive muscular dystrophy (*2*, *3*), it might be reasonable to examine the creatine phosphokinase activity in sera of this disease.

As shown in Table I, about seventy per cent of patients with progressive muscular dystrophy showed a remarkable increase in creatine phosphokinase activity. This is in sharp contrast with other subjects, in which a marked activity of the enzyme was not observed except for two cases with spinal progressive muscular atrophy of rather atypical type.

The formation of creatine phosphate by serum of progressive muscular dystrophy from creatine and ATP was also examined by other methods (*4*,

TABLE I

Creatine Phosphokinase Activity of Sera

	Number of cases	Enzymatic activities						
		Less than 0.3	Between 0.3 and 0.6	Between 0.6 and 1.0	Between 1.0 and 2.0	Between 2.0 and 5.0	Between 5.0 and 10.0	More than 10.0
Progressive muscular dystrophy	19	3	1	1	5	4	4	1
Spinal progressive muscular atrophy	10	7	1	0	1	0	1	0
Amyotrophic lateral sclerosis	5	5	0	0	0	0	0	0
Other neuromuscular diseases	24	22	2	0	0	0	0	0
Liver diseases	9	6	2	1	0	0	0	0
Other diseases	10	10	0	0	0	0	0	0
Normal	5	5	0	0	0	0	0	0

Figures in columns show the number of cases of respective activities. Activities are expressed as the number of μ moles of phosphocreatine formed per ml. of serum per hour at 38° and pH 8.8. Each ml. of reaction mixture A contained, 6 μ moles $MgCl_2$, 2 μ moles adenosine triphosphate, 30 μ moles tris-(hydroxymethyl)-aminomethane buffer (pH 8.8) and 0.2 ml. of serum; mixture B contained 24 μ moles creatine in addition to mixture A. Reactions were conducted at 38° for 30 minutes. Difference of so-called inorganic phosphate between mixture A and B was determined to measure the phosphocreatine produced (*1*).

5) than described in the legend of Table I; these gave almost the same values. The stability of the enzymic activity of the serum to ethanol treatment coincides with that of the creatine phosphokinase in skeletal muscle (*1*). Red blood corpuscles contained practically none of this enzyme. Therefore hemolysis does not significantly interfere with the measurement of this enzyme activity.

The above results suggest that creatine phosphokinase activity in serum is a valuable tool in studies on myopathies.

We wish to express our thanks to Prof. S. Okinaka and Prof. H. Kumagai for their encouragement and also to Dr. F. Ebashi and Dr. I. Ishiyama for their assistance.

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SULFATE REDUCTION IN CELL-FREE EXTRACTS OF DESULFOVIBRIO

A sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, reduces sulfate to sulfide with hydrogen or organic substances to obtain energy for its cellular activity. The precise enzymic mechanism of this process has not been elucidated, since the cell-free isolation of the complete reaction system has thus far met with only partial success of obtaining the enzyme systems catalysing the reduction of sulfite and thiosulfate to sulfide (*1*, *2*). This communication deals with a finding that the the complete process of the reduction of sulfate to sulfide can be achieved in a cell-free extract when ATP is supplied to the reaction system.

The sulfate reducing capacity of the cell-free extract obtained by grinding the cells of *Desulfovibrio* with alumina (*1*) was tested by adding methyl viologen as an intermediary electron carrier, and hydrogen as the H-donor. The hydrogen uptake was determined with Warburg's manometer,

TABLE I

Sulfate Reduction in the Cell-Free Extracts of Sulfate-Reducing Bacterium

	H_2 uptake (μ moles)	H_2 uptake due to sulfate addition (μ moles)	H_2S formed (μ moles)	H_2S formed due to sulfate addition (μ moles)
Complete system	10.3	7.0	2.95	2.05
−sulfate	3.3	—	0.90	—
−ATP	−0.1	—	0.15	—

The reaction mixtures contained: 16 μ moles tris-hydroxymethyl-aminomethane, pH 7.4, 2.0 μ moles K_2SO_4, 28 μ moles NaF, 1 μ mole methyl viologen, 10 μ moles ATP and crude extract of sulfate-reducing bacterium (0.7 mg. N) (*1*). Total volume was 1.4 ml. Gas phase was hydrogen; temperature 30°. The center wells of the vessels contained 0.2 ml. of 2 *N* NaOH. The reaction was initiated by the addition of ATP from the side arms, after the hydrogen uptake due to the reduction of methyl viologen had ceased.

and the hydrogen sulfide formed and absorbed in alkali in the center well of the vessel was determined by St. Lorant's method (*3*). The remarkable occurrence of the sulfate reduction caused by the presence of ATP may be apparant from the data presented in Table I. In the presence of ATP and absence of sulfate there occurred some hydrogen uptake and sulfide

formation, indicating the presence of a small amount of sulfate in the crude extracts. The molar amount of hydrogen absorbed and hydrogen sulfide evolved due to the added sulfate was 3.5 and 1.03 times, respectively, those found in the absence of added sulfate. These values indicate the occurrence of complete reduction of sulfate to sulfide according to the following equation:

$$SO_4^{--} + 4\,H_2 = S^{--} + 4\,H_2O$$

That ATP played an active role in this process was revealed by the fact that a sizable amount of inorganic phosphate was liberated during the reaction.

The enzyme system for this reaction seems to be contained chiefly in the supernatant of the extract (17,000×*g*, 15 minutes), although it was activated by the addition of the precipitate which was rich in hydrogenase (*4*). The precipitate with boiled supernatant did not absorb hydrogen in the presence of ATP. The sulfate reduction was not observed in a system consisted of the above-mentioned precipitate, methyl viologen and a sulfate-activating enzyme preparation obtained from liver (*5*) or yeast (*6*). The yeast enzyme preparation was found to contain a sulfite reductase in addition to the sulfate-activating system (*7*).

The active sulfate, 3′-phosphoadenosine-5′-phosphosulfate, has been stated to be an intermediate in the esterification of phenols (*5*). Recently it was shown that ATP is necessary in the reduction of sulfate to sulfite (*8*) or to sulfite (*9*) in the yeast extracts. The results of the present experiment show that, also in the case of sulfate-reduction in *Desulfovibrio*, the process of sulfate activation is an indispensable primary reaction.

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